

**CHARACTERIZATION OF CHRONIC ENTEROPATHIES IN DOGS
BY USE OF FECAL AND URINARY
N-METHYLHISTAMINE CONCENTRATIONS
AND SERUM METHYLMALONIC ACID CONCENTRATIONS**

A Dissertation

by

NORA BERGHOFF

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Veterinary Microbiology

Characterization of Chronic Enteropathies in Dogs
by Use of Fecal and Urinary
N-Methylhistamine Concentrations
and Serum Methylmalonic Acid Concentrations

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ABSTRACT

Characterization of Chronic Enteropathies in Dogs by Use of Fecal and Urinary
N-methylhistamine Concentrations and Serum Methylmalonic Acid Concentrations.

(August 2012)

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Non-invasive markers that are clinically useful for the diagnosis and monitoring of canine chronic enteropathies are scarce. The first aim of this study was to investigate the prevalence of cobalamin deficiency on a cellular level in dogs with chronic gastrointestinal disease by measuring serum methylmalonic acid (MMA) concentrations. Hypocobalaminemia has been associated with a negative outcome in dogs with chronic enteropathies, but the prevalence of cellular cobalamin deficiency is unknown. The second aim of this study was to determine the utility of fecal and urinary concentrations of N-methylhistamine (NMH) as a marker of gastrointestinal inflammation and disease activity in dogs with chronic enteropathies.

Serum MMA concentrations were measured in healthy control dogs to establish a reference interval, which was calculated to be 415-1,193 nmol/L. Measurement of MMA concentrations in 555 serum samples from dogs with varying cobalamin concentrations showed a significant increase ($p < 0.05$) in dogs with hypocobalaminemia. In a prospective group of 56 dogs with chronic enteropathies, 36% had decreased serum cobalamin concentrations, five of which (9% of 56 dogs) had increased serum MMA concentrations. We conclude that hypocobalaminemia is commonly seen in dogs with chronic gastrointestinal disease, but does not always appear to be associated with cellular cobalamin deficiency.

In 47 dogs with chronic enteropathies, fecal and urinary NMH concentrations were increased in 21% and 27%, respectively, indicating that mast cell degranulation

plays a role in a subset of dogs with chronic enteropathies. However fecal and urinary NMH concentrations did not correlate with each other, or with the clinical activity index. Urinary NMH concentrations correlated significantly with serum CRP concentrations, and were also significantly associated with severity of duodenal mucosal inflammation ($p=0.008$). The lack of correlation with the clinical activity index suggests that degranulation of mast cells only plays a role in some dogs with chronic enteropathies. Also, these results suggest that NMH alone may not be a good marker for clinical disease activity in dogs with chronic enteropathies. Due to its linear association with serum CRP and severity of mucosal inflammation, urinary NMH concentrations may be a better marker of intestinal inflammation than fecal NMH concentrations.

DEDICATION

To my family and friends.

ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. Jörg Steiner, Dr. Jan Suchodolski, Dr. Joanne Mansell, Dr. Mike Willard, and Dr. Karin Allenspach. Their expertise has been very helpful for the completion of my PhD. Special thanks go to my committee chair Dr. Jörg Steiner for allowing me to perform my graduate work in the GI Lab.

I would also like to thank the team of service and research technicians, graduate students, and student workers in the GI lab, many of whom have helped by processing samples and running assays, or by lending their support.

A very special thank you goes to all the veterinarians, dog owners, and their dogs that were part of my research study. Without them, none of this would have been possible, and I am extremely grateful for their help and participation.

Last, but most certainly not least, a very warm thank you goes to my family and all my dear friends who have supported and encouraged me over the years, during good and tough times. You know who you are. You are much appreciated and loved.

NOMENCLATURE

ALP	Alkaline phosphatase
α_1 -PI	Alpha ₁ -proteinase inhibitor
ALT	Alanine aminotransferase
ARD	Antibiotic-responsive diarrhea
AST	Aspartate aminotransferase
CCECAI	Canine chronic enteropathy clinical activity index
CRP	C-reactive protein
cTLI	Canine trypsin-like immunoreactivity
d3-MMA	Trideuterated methylmalonic acid
d3-NMH	Trideuterated N-methylhistamine
ELISA	Enzyme-linked immunosorbent assay
EPI	Exocrine pancreatic insufficiency
FRD	Food-responsive diarrhea
GC/MS	Gas chromatography/mass spectrometry
GGT	Gamma-glutamyltransferase
HNMT	Histamine N-methyltransferase
IBD	Idiopathic inflammatory bowel disease
MMA	Methylmalonic acid
MSD	Mass selective detector (mass spectrometer)
MTBSTFA	N-methyl-N- (tert-butyldimethylsilyl)-trifluoroacetamide
m/z	Mass-to-charge ratio
NMH	N-methylhistamine
PFPA	Pentafluoropropionic anhydride
PLE	Protein-losing enteropathy
RIA	Radioimmunoassay
r_s	Spearman's rank correlation coefficient
Spec cPL®	Serum canine pancreas-specific lipase

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1. INTRODUCTION

1.1 CANINE CHRONIC ENTEROPATHIES

Chronic enteropathy is a term used for diseases of the intestines regardless of etiology and pathogenesis. Chronic enteropathies are commonly seen in the dog. Objective laboratory tests that are clinically useful for the diagnosis and monitoring of canine chronic enteropathies are scarce. Specifically, non-invasive markers for gastrointestinal inflammation in the dog that have been shown to be clinically useful are currently not widely available. Likewise, tests that may help distinguish the different forms of chronic enteropathies are lacking, making it nearly impossible to predict a response to treatment in affected dogs.^{1,2}

Differentiating the various types of canine chronic enteropathies is complicated due to a current lack of uniformity among authors in defining these entities. Particularly, the term “inflammatory bowel disease” is not well defined in veterinary medicine. Technically, inflammatory bowel disease describes any condition associated with an inflammatory infiltration of the GI tract.³ Idiopathic inflammatory bowel disease (IBD), however, is any condition associated with presence of chronic (>3 weeks) gastrointestinal signs and an inflammatory infiltration of the GI tract where a specific etiology cannot be identified. Thus, a distinction should be made between idiopathic IBD, in which the cause is unknown, and those types of gastrointestinal inflammation for which an underlying cause can be determined.³

This dissertation follows the style of *American Journal of Veterinary Research*.

Frequently, the response to treatment is used to allow for distinction of different types of enteropathies, such as food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), and steroid-responsive diarrhea (SRD),² all of which can be features of idiopathic IBD. Treatments are applied in a sequential fashion, starting with a dietary trial, followed by antibiotic therapy if there is a lack of response to diet, and finally, treatment with anti-inflammatory drugs, if response to previous treatments was inadequate.²

The reason for a response to these therapeutic approaches, or lack thereof, in dogs with idiopathic IBD is currently unknown.⁴ Many patients with chronic enteropathies that respond to a change in diet can later be switched back to their original diet.¹ This would not be possible if a true adverse food reaction, such as food allergy or food intolerance, was the cause of the enteropathy.⁵ Instead, it has been suggested that improved nutrient bioavailability resulting from an intestinal or elimination diet, or additives such as fructooligosaccharides and/or higher n3-n6 fatty acid ratios may be partially responsible for the favorable response to a change in diet in these dogs.⁵

Thus, diagnosis of canine chronic enteropathy and idiopathic IBD currently remains a diagnosis of exclusion (i.e., ruling out other causes of intestinal disease and inflammation). This situation highlights the need for laboratory tests that would help differentiate different types of chronic enteropathies.

Pathogenesis of idiopathic IBD is generally believed to be multifactorial.² A currently accepted hypothesis in humans, dogs, and cats is that of a dysregulation of the gastrointestinal immune system in genetically susceptible individuals, which may lead to aberrant responses towards luminal microbial or dietary antigens.⁶⁻⁹ Inappropriate immune regulation may cause loss of tolerance of commensal bacteria, and possibly other antigens (e.g., dietary components).^{2,9} This multifactorial pathogenetic theory suggests that different avenues should be explored so that laboratory tests with clinical utility can be discovered.

1.2 PREVIOUSLY INVESTIGATED MARKERS OF DISEASE FOR DOGS WITH CHRONIC ENTEROPATHIES

Several molecules that may play a role in the pathogenesis of IBD have been investigated as potential disease markers in dogs with chronic enteropathies. These include toll-like receptors, cytokines, and markers of inflammation:

Toll-like receptors - Toll-like receptors (TLR) are important in intestinal immune regulation. They recognize microbe-associated molecular patterns (MAMPs) present on microbial organisms. Toll-like receptors are expressed by a variety of cell types including epithelial cells as well as cells of the innate immune system (e.g., macrophages, dendritic cells, mast cells).^{10,11} In humans, mutations of TLRs have been found to be associated with IBD, suggesting that they play a role in regulating mucosal homeostasis.¹² The TLRs studied in dogs to date are TLR2, TLR4, TLR5, and TLR9, which recognize lipoproteins, lipopolysaccharide, flagellin, and unmethylated bacterial or viral CpG DNA, respectively.¹¹ Binding of the respective ligand to its TLR causes translocation of the transcription factor Nuclear Factor- κ B (NF- κ B) into the nucleus, which subsequently initiates transcription of genes encoding pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6.¹¹

One study in dogs evaluated mRNA expression of canine TLR2, TLR4, and TLR9, and found all three TLRs to be upregulated in dogs with chronic enteropathies.¹³ Interestingly, expression profiles did not change after dogs were treated and had improved clinically, supporting the hypothesis of a genetic predisposition that caused an aberrant immune response. Another study found increased TLR2 expression in dogs with idiopathic IBD, but TLR4 expression was not different from controls.¹⁴ A third investigation found increased expression of TLR4 in German Shepherd dogs (GSD) with chronic enteropathies, whereas TLR5 was decreased and TLR2 and TLR9 were unchanged.⁸ Furthermore, a risk-associated haplotype of TLR5 has recently been identified in German Shepherd dogs and has been shown to be significantly hyper-responsive to flagellin.¹⁵ The authors hypothesized that this may be partially responsible for the exaggerated inflammatory response seen in dogs with idiopathic IBD.¹⁵

These reports demonstrate that an aberrant expression of TLRs in dogs with chronic enteropathies occurs and warrants further investigation to understand the exact pathogenetic mechanisms of the disease. However, due to discrepant results between the different studies, no single TLR or combination of TLRs can currently be recommended as a diagnostic marker for dogs with chronic enteropathies. Also, the need for intestinal biopsies limits the clinical utility of these tests as a diagnostic aid.

Cytokines - Mucosal mRNA expression of inflammatory cytokines as an indirect measure of the intestinal immune response is another area of investigation. In humans, various cytokines have been associated with IBD.⁶ More specifically, TNF- α and IL-12p40 have been implicated in the pathogenesis of Crohn's disease in humans. Several studies have been carried out in dogs, investigating a variety of cytokines.¹⁶⁻¹⁸

A study by Peters et al. looked at mRNA expression of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, IFN- γ , TNF- α , and TGF- β in dogs with chronic enteropathies and healthy control dogs. Investigators were unable to find significant differences in cytokine mRNA expression between the two groups of dogs.¹⁶ Another investigation showed that dogs with lymphocytic-plasmacytic colitis had a higher expression of IL-2 and TNF- α .¹⁷ However, the opposite was shown in a study by Jergens et al.¹⁸ In this report, a meta-analysis of four studies was performed, and the authors concluded that only IL-12 was consistently overexpressed in dogs with idiopathic small intestinal IBD.¹⁸

Overall, there is a lack of an obvious pattern of cytokines that are differentially expressed in dogs with chronic enteropathies. This may in part be due to differences in methodology, sampling populations, and types or stages of disease between the studies. There is some evidence that IL-12 may be overexpressed in dogs with chronic enteropathies, which would mirror reports in human patients with IBD.^{6,18}

The ability to measure cytokine concentrations in serum or plasma samples would be more clinically relevant than determination of mRNA expression profiles in intestinal biopsies, as it would be less invasive and results could be obtained more easily and less invasively. Unfortunately, studies determining the clinical utility of cytokines as

a marker of disease in dogs with IBD are lacking. One study evaluated use of a serum TNF- α assay in dogs with IBD, but TNF- α concentrations were not different in dogs with IBD compared to the reference population.¹⁹ Thus, further investigations are required to determine which cytokines play a role in the pathogenesis of canine idiopathic IBD, and if any cytokines may be clinically useful, especially when measured in serum or plasma.

Inflammatory markers - pANCA and C-reactive protein have been investigated as markers of inflammation in dogs with chronic enteropathies.

pANCA - Perinuclear antineutrophilic cytoplasmic antibodies (pANCA) are used as a marker of inflammation in human patients with IBD to distinguish patients with ulcerative colitis from those with Crohn's Disease. pANCAs are autoantibodies that produce a characteristic perinuclear staining pattern in granulocytes when stained with immunofluorescence detection methods.²⁰ In human patients with IBD, pANCAs can be identified in the serum of about 50% to 80% of patients with ulcerative colitis, whereas most patients (70%–90%) with Crohn's disease are negative.^{20,21}

In dogs, an assay for the detection of pANCA had a variable sensitivity from 23% to 51% and a specificity of 83% to 95% for canine IBD.²⁰⁻²² Thus, there appears to be an association between the detection of pANCA and IBD in dogs, but because of its poor sensitivity, the assay may not be useful as a screening test for IBD.²⁰ Interestingly, one study found a higher rate of pANCA positive samples from dogs with FRD (62%) when compared with those from dogs with idiopathic IBD (23%).²¹ Furthermore, 20 of 21 Soft-Coated Wheaten Terriers with protein-losing enteropathy and nephropathy (PLE and PLN, respectively) were positive for pANCA.²³ In most of these dogs, pANCA was positive one to two years before onset of hypoalbuminemia, and the assay was able to predict PLE and/or PLN with a sensitivity of 95% and specificity of 80%. Thus, this test may prove helpful in early diagnosis and, once preventative strategies have been identified, in early treatment of affected dogs.²³

C-reactive Protein - C-reactive Protein (CRP) is a positive acute phase protein, meaning that its serum concentration increases in response to an inflammatory stimulus,

regardless of the affected organ. While this response is not specific to the intestinal tract and can be caused by inflammatory conditions throughout the body, one study has shown significantly increased serum CRP concentrations in dogs with moderate to severe IBD, when compared to a group of healthy control dogs.²⁴ Another investigation demonstrated that serum CRP concentrations in dogs with IBD decrease significantly after treatment.²⁵ However, other investigators were not able to detect a difference in serum CRP concentrations between healthy dogs and dogs with IBD.^{1,19}

Therefore, while measurement of serum CRP concentrations is of limited use in diagnosis of canine IBD and assessment of clinical disease activity, it may be beneficial in evaluating response to treatment in individual patients.

1.3 NEW MARKERS WITH POTENTIAL TO AID IN THE DIAGNOSIS AND MONITORING OF CANINE CHRONIC ENTEROPATHIES

Based on the results obtained from the aforementioned studies, it is clear that clinically useful markers for diagnosis and monitoring of canine chronic enteropathies are still lacking. Ideally, a test should be non- or minimally invasive and thus should be using blood, urine, or fecal samples, as opposed to intestinal biopsies. A test should also be quick and convenient to use, providing a fast turnaround time. The following describes two new markers with potential usefulness for the diagnosis and monitoring of canine chronic enteropathies.

Serum methylmalonic acid concentration as a marker of cobalamin deficiency on a cellular level in dogs - Cobalamin (vitamin B₁₂) is a water soluble vitamin that requires a complex set of physiologic mechanisms for intestinal absorption.^{26,27} Protein-bound cobalamin is released after partial digestion of the protein in the stomach, but immediately binds to haptocorrin (aka R-protein or cobalophilin). In the small intestine, a higher pH and action of pancreatic proteases result in dissociation of haptocorrin from cobalamin, and free cobalamin binds to intrinsic factor. The cobalamin-intrinsic factor complex is then absorbed by receptors on ileal enterocytes.

In dogs, cobalamin deficiency usually develops in patients with exocrine pancreatic insufficiency or distal small intestinal disease, or may be caused by genetic defects in certain breeds. Exocrine pancreatic insufficiency (EPI) frequently causes hypocobalaminemia in dogs, with a reported prevalence of up to 82%,²⁸ because the majority of intrinsic factor in dogs is believed to be synthesized by pancreatic acinar cells, which is therefore deficient in patients with EPI.²⁹ Long-standing and severe ileal disease can lead to damage or decreased expression of cobalamin receptors, reducing cobalamin absorption and ultimately leading to cobalamin deficiency.²⁶

Studies in cats with gastrointestinal disease have shown a prevalence of cobalamin deficiency from 17% to 61%.^{30,31} In dogs with chronic gastrointestinal disease, the reported prevalence in three studies appears to be lower at 6%, 19%, and 31%, respectively.^{1,25,32}

Cobalamin is an essential co-factor for the methylmalonyl CoA mutase enzyme systems.^{27,33-35} L-methylmalonyl CoA mutase converts L-methylmalonyl CoA to succinyl CoA. Because this reaction requires adenosyl-cobalamin, patients with cobalamin deficiency at the cellular level have a reduced ability to perform this conversion, and the metabolism of methylmalonyl CoA is shifted towards an alternate pathway. This pathway is catalyzed by D-methylmalonyl CoA hydrolase and results in the production of methylmalonic acid (MMA).³⁶ Because production of MMA occurs when cellular stores of adenosyl-cobalamin are inadequate, serum MMA concentrations may be a useful marker for cobalamin deficiency on a cellular level.^{34,36,37}

Thus, measurement of the serum MMA concentration may add diagnostic value in some canine patients with chronic enteropathies, since serum cobalamin concentrations do not always reflect the cellular cobalamin status.^{37,38} In fact, cellular cobalamin status may be more important than serum cobalamin concentrations because cobalamin-dependent biochemical reactions take place intracellularly. One previous study had shown that cats with hypocobalaminemia had significantly increased serum MMA concentrations when compared to healthy cats.³³

In humans, serum creatinine and MMA concentrations have been shown to correlate because excretion of MMA occurs primarily through the kidneys. Thus, renal insufficiency may increase serum MMA concentrations unrelated to cobalamin deficiency.³⁹⁻⁴¹ This has not yet been investigated in dogs, but increased serum MMA concentrations in patients with renal insufficiency may need to be interpreted cautiously.

While the serum cobalamin concentration is often measured in dogs with chronic small intestinal disease, the relationship between serum cobalamin and MMA concentrations in dogs has not been reported. The proportion of dogs with hypocobalaminemia that have concurrent cobalamin deficiency on a cellular level is unknown. Furthermore, it is not known what proportion of dogs with normal serum cobalamin concentrations may have evidence of cobalamin deficiency on a cellular level.

Finally, a recent study has suggested that hypocobalaminemia was a risk factor for negative outcome in dogs with chronic enteropathies, and may indicate refractoriness to treatment.¹ However, serum MMA concentrations as an indicator of cobalamin deficiency on a cellular level have not previously been investigated in dogs with chronic enteropathies. Therefore the number of dogs with evidence of cobalamin deficiency may be higher than previously assumed since laboratory assessment was based on serum cobalamin concentrations.

N-Methylhistamine as a marker of intestinal inflammation and mucosal mast cell degranulation - Mast cells are increasingly recognized as an important sentinel cell type for reactivity of the innate and adaptive immune system.¹⁰ They express a variety of TLRs and thus can recognize a variety of ligands, including microbial proteins.¹¹ They also possess the ability to present antigen,¹⁰ making them a potentially significant part of the intestinal immune system. Mast cells release a variety of inflammatory mediators, including histamine, tryptase, TNF- α , TGF- β , and a number of pro- and anti-inflammatory cytokines.⁴² Therefore, they can influence intestinal function and also participate in acute as well as chronic inflammatory conditions of the intestinal tract.⁴³

In humans, mast cells can be found throughout all layers of the intestinal wall of healthy individuals.⁴³ Furthermore, studies indicate that mast cell density in the human colon decreases from the cecal region towards the rectum.⁴⁴ In healthy dogs, the density of mast cells appears to increase from villus tip to crypt while no significant difference was found between different regions of the intestinal tract.⁴⁴

Increased numbers of mucosal mast cells have been observed in a variety of studies investigating human patients with IBD.^{42,43,45-47} High numbers of mast cells are considered suggestive of active disease and inflammation.⁴² Several studies have shown that most of these mast cells are not intact but rather degranulated.⁴³ This indicates that participation in the inflammatory process has taken place and further suggests that mast cells play a role in the pathophysiology of IBD.⁴³ This assumption is supported by the fact that an increased release of histamine in the intestinal mucosa as well as into the intestinal lumen has been documented at sites with an increased mast cell density.⁴³ Because histamine mediates pro-inflammatory effects on other immune cells as well as nerve and smooth muscle cells, it may also contribute to some of the clinical signs observed in patients with IBD.⁴³

Histamine is a biogenic amine and a well-documented inflammatory mediator. It is primarily stored in mast cells, and to a much lesser extent in basophils, enterochromaffin cells, and platelets.⁴⁸ Histamine has been proposed as a marker of mast cell degranulation.⁴² However, after release from mast cells, histamine is rapidly metabolized by either of two pathways, which may interfere with its measurement.

The first pathway yields imidazole acetic acid via the diamine oxidase enzyme system. The second pathway leads to production of N-methylhistamine (NMH) via the histamine N-methyltransferase (HNMT) enzyme system.⁴⁹ In humans, endogenously produced histamine is preferentially metabolized through the N-methyltransferase pathway.⁴³ Therefore, it has been suggested to use the stable metabolite NMH as a marker for mast cell degranulation and gastrointestinal inflammation in people.

Increased urinary NMH concentrations have been documented in human patients with active Crohn's disease and ulcerative colitis.^{43,50,51} One study showed that urinary

concentrations of NMH correlate with endoscopic severity indices,⁴³ while another documented a correlation with disease activity.⁵⁰ Furthermore, urinary NMH concentrations have been shown to correlate with serum CRP concentrations.⁵¹ The correlation between urinary NMH concentrations and clinical disease activity suggests that mast cells become activated only during flare-ups, but do not release histamine at other times.⁵⁰ Thus, the measurement of NMH in urine may provide a useful tool for monitoring of disease activity in dogs with chronic enteropathies.

Several studies in dogs with chronic enteropathies have described the distribution of mucosal mast cells in the intestinal tract.⁵²⁻⁵⁴ One study reported a reduced number of lamina propria mast cells in dogs with idiopathic IBD when tissue was stained with toluidine blue.⁵² Another study found increased numbers of mast cells throughout the stomach and small intestine in dogs with idiopathic IBD. However, in this study, mast cells were detected using immunohistochemistry (IHC) staining for tryptase.⁵³ A third study used both metachromatic and IHC staining procedures and demonstrated decreased mast cells using both staining procedures in dogs with lymphoplasmacytic enteritis.⁵⁴

The different staining methods for mast cells may be important. Metachromatic stains work properly only on intact mast cells, whereas degranulated mast cells may not be visible. Immunohistochemical stains for mast cell enzymes such as tryptase or chymase, however, appear to function even in degranulated mast cells because residues of these enzymes are still present within the cell.⁵⁴ The majority of mast cells detected in affected tissues of human patients with IBD were degranulated; therefore, the same might occur in canine IBD patients. Depending on the staining method, mast cells may not have been detected.

Aside from differences between metachromatic or IHC staining, intestinal mucosal mast cells have been reported to be sensitive to formalin fixation.⁵⁵ Thus, length of fixation and type and concentration of fixative used may have had an impact on the outcome of these studies. Furthermore, different stages of inflammation in the dogs studied may also have influenced the findings (i.e., some dogs may have had more

pronounced mast cell degranulation compared to others). It remains to be determined whether dogs with IBD have an overabundance of mucosal mast cells as has been reported in human IBD patients.

An assay for measurement of NMH in canine urine and fecal samples has recently been described.⁵⁶ Fecal NMH concentrations were shown to be increased in Norwegian Lundehunds with gastrointestinal disease when compared to healthy control dogs.⁵⁷ Mucosal mast cell staining was not performed in these dogs, and direct correlations with fecal NMH concentrations could not be evaluated. However, Norwegian Lundehunds have gastrointestinal inflammation, which is usually characterized by lymphoplasmacytic infiltration and is generally accompanied by intestinal lymphangiectasia.⁵⁸ Therefore, these dogs may have had high mucosal mast cell counts causing an increased histamine release. Furthermore, increased fecal NMH concentrations have been detected in some Soft Coated Wheaten Terriers with gastrointestinal disease.⁵⁹ Increased mast cell degranulation has been implicated as a potential contributing factor in the development of PLE and PLN in Soft Coated Wheaten Terriers.⁶⁰

Because the etiology of canine FRD is unknown, it is possible that FRD comprises chronic enteropathies with different etiologies, such as true food allergies (i.e., reaction mediated by the immune system), dietary intolerance (i.e., reaction not mediated by the immune system), and others that are not caused by dietary factors per se, but respond to special foods.⁵ IgE-mediated degranulation of mast cells can cause severe clinical signs, because mast cells generally degranulate more rapidly when triggered by IgE and antigens than when triggered by other factors.¹¹ Thus, highly increased NMH concentrations in fecal or urine samples may be expected in dogs with food allergies or hypersensitivities. Food hypersensitivities have been documented in Soft Coated Wheaten Terriers,⁶¹ and increases in fecal NMH concentrations that have previously been observed may also be attributed to such an etiology.

1.4 HYPOTHESES AND OBJECTIVES

The **hypotheses** for this study are that:

1. Cobalamin deficiency on a cellular level occurs commonly in dogs with chronic enteropathies, and serum methylmalonic acid can serve as a marker of cellular cobalamin deficiency.

2. Fecal and urinary N-methylhistamine concentrations reflect the degree of intestinal mucosal mast cell degranulation in dogs with chronic enteropathies.

The **objectives** to prove or disprove the aforementioned hypotheses are:

1. To measure methylmalonic acid concentrations in serum samples from dogs with varying serum cobalamin concentrations in order to determine the relationship between serum cobalamin and methylmalonic acid concentrations in dogs.

2. To determine the prevalence of cobalamin deficiency on a cellular level in dogs with chronic enteropathies by measuring serum methylmalonic acid concentrations, and to correlate these with the serum cobalamin concentrations in these dogs.

3. To measure N-methylhistamine concentrations in fecal and urine samples from dogs with chronic enteropathies and compare these to N-methylhistamine concentrations previously measured in healthy control dogs.

4. To correlate fecal and urinary N-methylhistamine concentrations with the clinical activity score (CCECAI) to determine its utility as a marker of active disease and clinical activity.

2. ASSOCIATION BETWEEN SERUM COBALAMIN AND METHYLMALONIC ACID CONCENTRATIONS IN DOGS*

2.1 INTRODUCTION

Cobalamin (Vitamin B₁₂) undergoes a complex mechanism of absorption, involving the pancreas and the distal small intestine.^{26,27} The two most common causes of hypocobalaminemia in dogs are exocrine pancreatic insufficiency (EPI) and severe, long-standing ileal disease. While EPI may cause hypocobalaminemia primarily through a lack of pancreatic intrinsic factor,²⁹ ileal disease can lead to a decrease in cobalamin receptors, and thus reduced absorption of cobalamin.²⁶

Cobalamin is an essential co-factor for the methylmalonyl CoA mutase system.^{27,33-35} L-methylmalonyl CoA mutase converts L-methylmalonyl CoA to succinyl CoA (**Figure 2.1**). Because this reaction requires adenosyl-cobalamin, patients with cellular cobalamin deficiency have a reduced ability to perform this conversion, and the metabolism of methylmalonyl CoA is shifted towards an alternate pathway. This pathway is catalyzed by D-methylmalonyl CoA hydrolase and results in production of methylmalonic acid (MMA).³⁶

Because production of MMA occurs when cellular stores of adenosyl-cobalamin are inadequate, serum MMA concentrations may be a useful marker of cobalamin deficiency on a cellular level.^{34,36,37} This may be of value because the serum cobalamin concentration does not always reflect the cellular cobalamin status.^{37,38} However, cellular cobalamin concentrations may be more important than serum cobalamin concentrations, because cobalamin dependent biochemical reactions take place intracellularly.

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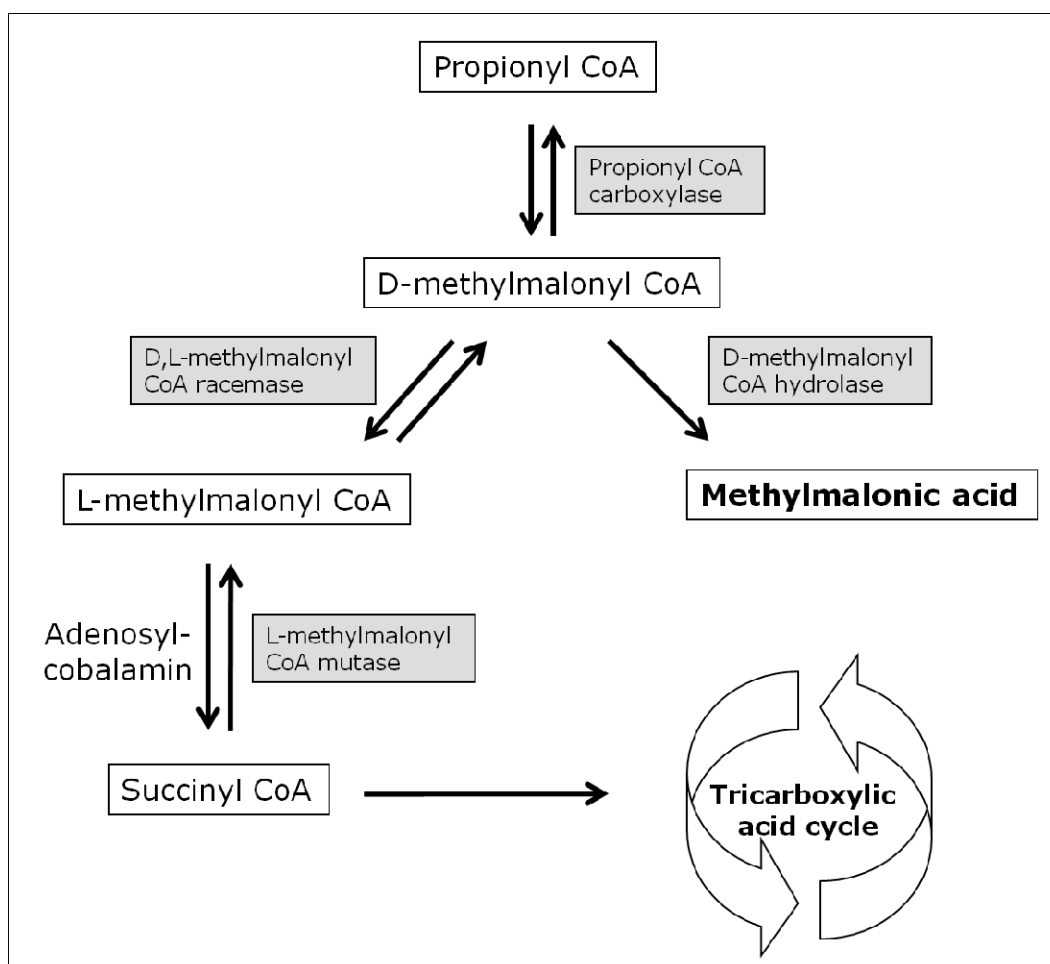


Figure 2.1 – Pathway of MMA formation. Propionyl CoA is converted to D-methylmalonyl CoA, which can subsequently enter two different pathways. Depicted on the left side, the racemate L-methylmalonyl CoA undergoes transformation to succinyl CoA. This reaction is dependent on adenosyl-cobalamin. Thus, during states of cobalamin deficiency, this pathway cannot be fully utilized. Instead, the reaction illustrated on the right side is favored. This alternative pathway irreversibly converts D-methylmalonyl CoA into MMA without the need for cobalamin.

In humans, serum creatinine and MMA concentrations have been shown to correlate, because excretion of MMA occurs primarily through the kidneys. Thus, renal insufficiency may increase serum MMA concentrations unrelated to cobalamin deficiency.³⁹⁻⁴¹ This has not yet been investigated in dogs, but increased serum MMA concentrations in patients with renal insufficiency may need to be interpreted with caution.

While the serum cobalamin concentration is often measured in dogs with chronic small intestinal disease, the relationship between serum cobalamin and MMA concentrations in dogs has not been reported, and the portion of hypocobalaminemic dogs with concurrent cellular cobalamin deficiency is unknown. Furthermore, the number of dogs with normal serum cobalamin concentrations but cellular cobalamin deficiency is unknown.

We hypothesized that increased serum MMA concentrations will be present in dogs with hypocobalaminemia. Therefore, the aims of this study were: (1) to determine a reference interval for MMA concentrations in canine serum, and (2) to evaluate the association between serum MMA and cobalamin concentrations in dogs.

2.2 MATERIALS AND METHODS

Samples for determination of the MMA reference interval - Blood samples were collected from 43 healthy, client owned dogs. Owners were required to fill out a questionnaire to help exclude presence of underlying diseases. A brief physical examination was performed by a veterinarian and all dogs appeared healthy. Blood was obtained by jugular venipuncture, placed into blood collection tubes without additive (Monoject, TYCO Healthcare Group LP), allowed to clot at room temperature, and centrifuged at $1,500 \times g$. Serum was decanted from the clot and stored at -80°C until further use. The Clinical Research Review Committee at the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University approved the protocol for use of client-owned dogs enrolled in this part of the study (CRRC#: 08-30).

Samples for evaluating the association between serum cobalamin and MMA concentrations - Leftover canine serum samples from submissions to the Gastrointestinal Laboratory were collected over a period of 4 months (June – September 2008). To be included in this study, a sample had to be submitted for a cobalamin assay, and a serum volume sufficient to perform MMA analysis had to be available. Sample stores were searched for subsequent accessions that fulfilled these criteria, until a

sufficient number of samples were collected over a wide range of cobalamin concentrations. No exclusions were made based on underlying disease or breed, because these were deemed irrelevant for the purpose of this study.

A total of 555 serum samples were identified, all of which constituted residual samples that would otherwise have been discarded. Samples were stored at -20°C from the time the cobalamin assay was performed until serum MMA analysis. Internal laboratory data show that serum MMA is stable for at least one year when frozen (data not shown). Thus, no loss of analyte over the 4-month study period could be expected. For statistical analysis, dogs were divided into groups 1 to 10 according to their serum cobalamin concentration (**Table 2.1**).

Serum cobalamin assay - Serum cobalamin concentrations were determined using a competitive binding chemiluminescence assay.^a Our laboratory's reference interval for this assay was 251-908 ng/L. The lower and upper detection limits of the assay were 150 and 1,000 ng/L, respectively.

Serum creatinine assay - Serum creatinine concentrations were measured in all samples with sufficient sample volume ($n = 542$), using an automated clinical chemistry analyzer^b with an enzymatic assay method.^c The reference interval for serum creatinine was 0.5-1.4 mg/dL. Samples were measured throughout nine assay runs. The intra-assay CV% was 0.79%, and the inter-assay CV% was 1.11%.

Table 2.1 – Serum MMA concentrations in dogs with varying cobalamin concentrations. Dogs were grouped based on their serum cobalamin concentrations. The table shows the median, minimum, and maximum serum MMA concentrations (nmol/L) in each of the 10 groups. Results are shown for all 555 samples evaluated, as well as for those 488 dogs that had a serum creatinine concentration below the upper limit of the reference range (≤ 1.4 mg/dL). The far right column shows the percentages (and 95% confidence intervals (CI) of the percentages) of dogs with serum MMA concentrations above the reference interval in each group (of all 555 dogs).

		MMA (nmol/L)								% Dogs with serum MMA above the reference interval
		All dogs				Dogs with creatinine ≤1.4 mg/dL				
Group	Cobalamin (ng/L)	n	Median	Min	Max	n	Median	Min	Max	% (95% CI)
1	<150	72	1,328.0	492.0	24,764.6	60	1,289.0	492.0	24,764.6	63% (51-73%)
2	151-250	81	1,033.0	469.1	3,002.6	69	1,038.0	469.1	2,739.8	31% (21-41%)
3	251-350	52	759.5	378.9	4,063.6	46	786.0	378.9	4,063.6	19% (9-30%)
4	351-450	50	741.9	397.2	2,446.9	40	740.8	397.2	1,955.6	16% (6-26%)
5	451-550	60	710.2	405.0	1,396.1	54	692.9	405.0	1,373.6	10% (2-18%)
6	551-650	49	690.5	398.6	1,733.3	44	640.3	398.6	1,733.3	14% (5-24%)
7	651-750	50	730.3	391.7	1,807.4	48	718.0	391.7	1,515.2	6% (-1-13%)
8	751-850	52	820.9	332.0	1,523.1	47	820.1	332.0	1,477.8	10% (2-18%)
9	851-950	49	673.6	343.1	1,765.0	47	652.7	343.1	1,765.0	10% (2-19%)
10	951-1,000	40	733.0	423.1	1,739.0	33	712.5	423.1	1,739.0	13% (2-23%)
		555				488				

Methylmalonic acid assay - Methylmalonic acid was measured using a stable isotope dilution gas chromatography/mass spectrometry (GC/MS) method as previously described.³³ Assay performance was verified using standards prepared with pure MMA^d in serial dilution from 16,000 to 62.5 nmol/L. The upper limit of the working range for the assay was 16,000 nmol/L. Samples with concentrations of 16,000 nmol/L or higher were rerun in a dilution to obtain an accurate measurement. MMA was quantified using the area under the curve for MMA and the internal standard.^e

Statistical analysis - Data were analyzed using statistical software packages.^{fg} Data sets were tested for normal distribution using a D'Agostino & Pearson omnibus test. The reference interval for serum MMA was calculated using the central 95th percentile. Serum MMA and creatinine concentrations in the 10 groups of dogs were compared using a Kruskal-Wallis test with Dunn's Multiple Comparison test. A χ^2 -test was used to calculate the odds of finding increased serum MMA concentrations in dogs with serum cobalamin below and within the reference interval. A χ^2 -test for trend was calculated for the 10 groups of dogs with cobalamin concentrations in ascending order. This test takes the sequential order of the groups into account, and was used to determine if there was a linear relationship between decreasing serum cobalamin concentrations and increasing serum MMA concentrations. The correlation between serum cobalamin and MMA concentrations was calculated using Spearman's rank correlation. The effects of age, serum folate, cobalamin, and creatinine concentrations on serum MMA concentrations were evaluated using a multiple linear regression model. Statistical significance for all tests was set at $P < 0.05$.

2.3 RESULTS

Reference interval for canine serum MMA concentration - The reference interval for canine serum MMA concentration determined in the 43 control dogs was 414.7-1,192.5 nmol/L (**Figure 2.2**). The mean serum MMA concentration was 697.8 nmol/L (95% confidence interval (CI): 625.7-769.9 nmol/L). Mean serum cobalamin

concentration was 539 ng/L (95% CI: 492-585 ng/L) and was within the reference interval in all control dogs.

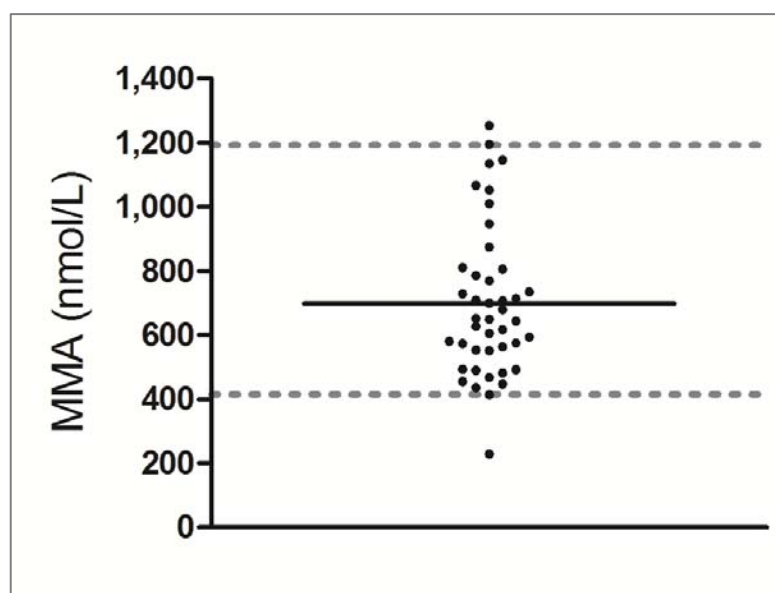


Figure 2.2 – Reference interval for serum MMA concentration. The reference interval based on the central 95th percentile was calculated as 414.7-1,192.5 nmol/L and is depicted by the two dotted lines. The mean MMA concentration in the 43 dogs was 697.8 nmol/L and is represented by the solid black line.

Serum creatinine concentrations - Serum creatinine concentrations were measured in 542/555 dogs (97.7%). In 13 dogs, serum volumes were insufficient for analysis. Serum creatinine concentrations were increased (>1.4 mg/dL) in 54/542 dogs (10%). Medians and ranges for serum creatinine concentrations in the 10 groups of dogs are shown in **Table 2.2**.

Table 2.2 – Serum creatinine concentrations in 542 of 555 dogs. Serum creatinine concentrations could not be measured in 13 dogs due to insufficient serum volumes.

Group	Cobalamin (ng/L)	Creatinine (mg/dL)			
		n	Median	Min	Max
1	<150	72	0.9	0.3	3.1
2	151-250	79	1.1	0.3	2.5
3	251-350	50	1.0	0.3	1.9
4	351-450	47	1.0	0.5	2.3
5	451-550	59	0.9	0.1	2.8
6	551-650	48	0.9	0.3	1.9
7	651-750	49	0.8	0.3	4.1
8	751-850	51	0.8	0.3	3.3
9	851-950	49	0.8	0.1	1.5
10	951-1,000	38	0.8	0.4	4.6
		542			

Association between serum cobalamin and serum MMA concentrations - Medians and ranges for serum MMA concentrations in the 555 serum samples are shown in **Table 2.1** and **Figure 2.3**. Dogs in groups 1 and 2 (cobalamin < 150 and < 151 -250 ng/L, respectively) had significantly higher serum MMA concentrations than dogs of all other groups ($P < 0.05$), with exception of groups 4 and 8 (cobalamin of 351-450 and

751-850 ng/L, respectively), which were not different from group 2. These results did not change when dogs with a serum creatinine concentration above the reference interval (> 1.4 mg/dL) were excluded from analysis.

Percentages (and 95% CI) of dogs with MMA concentrations above the upper limit of the reference interval in groups 1 through 10 are shown in **Table 2.1**.

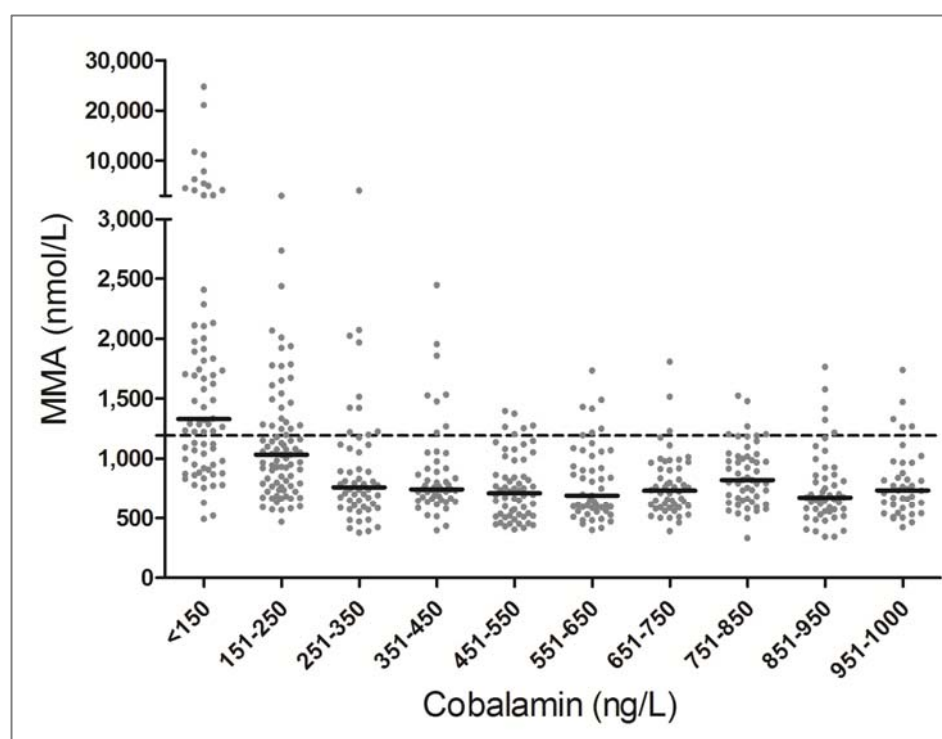


Figure 2.3 – Serum MMA concentrations in 10 groups of dogs (n=555) with cobalamin concentrations ranging from <150 ng/L to 1,000 ng/L. The dotted line represents the upper limit of the reference interval for canine serum MMA concentrations. Medians in each group are depicted by a solid black line.

Prevalence of cellular cobalamin deficiency as defined by methylmalonic acidemia in dogs with a serum cobalamin concentration below the reference interval (< 251 ng/L) was 46% (95% CI: 38-54%). In dogs with cobalamin concentrations within

the reference interval, prevalence of methylmalonic acidemia was 12% (95% CI: 9 - 15%). The odds ratio for dogs with subnormal cobalamin concentrations to have an increased serum MMA concentration ($> 1,192.5$ nmol/L) was 6.1 (95% CI: 3.9-9.4; $P < 0.0001$). The χ^2 -test for trend, testing for a linear trend between decreasing serum cobalamin and increasing serum MMA concentrations, was significant (Chi-square test statistic 58.3, $P < 0.0001$). A weak negative correlation was found between serum cobalamin and MMA concentrations (Spearman correlation coefficient ρ : -0.3860; 95% CI: -0.4566 to -0.3106; $P < 0.0001$).

Multiple regression analysis showed no effect of age, serum folate and serum creatinine concentrations on serum MMA concentrations. Serum cobalamin concentrations had a significant effect on serum MMA concentrations ($P < 0.0001$).

2.4 DISCUSSION

This is the first published study investigating the association between serum cobalamin and MMA concentrations in dogs. While negative correlation between serum cobalamin and MMA concentrations was not strong, the χ^2 -test for trend showed a linear increase in serum MMA concentration with a decreasing serum cobalamin concentration. This trend was expected based on the metabolic relationship of cobalamin and MMA.

Sixty-three percent of dogs with undetectable serum cobalamin concentrations (< 150 ng/L) had increased serum MMA concentrations, indicating cellular cobalamin deficiency. If the analysis is expanded to include all dogs with subnormal cobalamin concentrations (< 251 ng/L), an increased serum MMA concentration was observed in 46% of dogs. In cats, this prevalence is reported to be higher at 68%.³⁷ Hence, one may have expected a larger percentage of dogs to have increased serum MMA concentrations. It is unclear why there is such an interspecies discrepancy, though it is possible that there are distinct differences in the way cats and dogs utilize different pathways for cobalamin and MMA metabolism. Another potential reason may be the

higher prevalence of renal insufficiency in cats when compared to dogs.⁶² Ruaux et al. did not report serum creatinine concentrations; therefore, we cannot rule out that some of these cats had renal insufficiency, which may have contributed to increased serum MMA concentrations.

While the prevalence of methylmalonic acidemia in dogs with serum cobalamin concentrations within the reference interval was 12%, groups 3 (cobalamin of 251-350 ng/L) and 4 (cobalamin of 351-450 ng/L) had slightly higher percentages of affected dogs with 19% and 16%, respectively. These data imply that some dogs with serum cobalamin concentrations in the low normal range may have evidence of cobalamin deficiency on a cellular level. It is possible that some of these dogs were supplemented with cobalamin prior to analysis. Thus, it is unclear if there was a true deviation between serum MMA and cobalamin concentrations, or if some of the normal cobalamin concentrations may have been the result of cobalamin supplementation. It is possible that hypocobalaminemia was corrected by use of a cobalamin supplement, while it may take longer for the tissue to be replenished.

The lower limit of the serum cobalamin reference interval may not adequately reflect the cellular cobalamin status of all dogs. Dogs might have some degree of cobalamin deficiency on the cellular level even if serum cobalamin concentrations are within the reference interval. This lack of sensitivity of serum cobalamin concentrations to detect early cobalamin depletion is suspected to be a reason for discrepancies between serum MMA and cobalamin concentrations in humans,³⁴ where this phenomenon is termed ‘subtle’ or ‘mild preclinical’ cobalamin deficiency. It is defined as an increased concentration of metabolites (e.g., MMA) despite normal or low normal serum cobalamin concentrations.^{34,38} The significance of this condition in dogs is unknown. In humans, it is generally not associated with clinical signs, and it is unknown how many affected patients progress to clinical cobalamin deficiency.³⁸ Several disorders associated with cobalamin deficiency in humans (e.g., neuropathy, anemia) have also been reported in patients with subtle cobalamin deficiency, indicating that at least a subset of these patients apparently suffer physiological effects of cobalamin deficiency.³⁴

Therefore, while progression from the preclinical into the clinical state may be slow or even reversible, it may be advisable to treat these patients with cobalamin.⁴¹

In humans, an increased MMA concentration that responds to cobalamin supplementation with a significant reduction in serum MMA concentration is considered strong evidence, if not proof, of cobalamin deficiency.⁶³ A dose of 1,000 µg of cyanocobalamin is considered sufficient to trigger this decrease,³⁹ which may take up to 5 days to occur.⁶⁴ A study in cobalamin deficient cats with increased serum MMA concentrations showed that MMA concentrations decreased to within normal limits after the cats received four weekly doses of 250 µg.⁶⁵ To determine whether dogs with normal cobalamin and increased MMA concentrations are in fact cobalamin deficient, a similar cobalamin treatment trial will need to be conducted.

Limitations of this study include lack of clinical data in the dogs tested. It is unknown which clinical signs or diseases were present, or whether any dogs received cobalamin supplementation prior to testing. Supplementation could have increased serum cobalamin concentrations to within the reference interval, but may not have been sufficient to rectify a cellular deficiency. This might explain increased serum MMA concentrations in some dogs with normal serum cobalamin concentrations. However, it is unlikely that this significantly affected the results for dogs with low serum cobalamin concentrations.

Another limitation is renal insufficiency in some dogs. We measured creatinine in 542/555 dogs to evaluate if some of the increased serum MMA concentrations may have been due to impaired renal excretion of MMA. Serum MMA and creatinine concentrations correlate in humans,^{38,40} though some argue that this occurs only at creatinine concentrations >2.5 mg/dL.³⁹ Even then, increases in serum MMA are only moderate (up to 500 nmol/L) with creatinine concentrations as high as 7.9 mg/dL.³⁹ Our data showed no association between serum creatinine and MMA concentrations. Thus, renal insufficiency was considered unlikely to be contributing to increased serum MMA concentrations observed here.

Other than cobalamin deficiency and renal insufficiency, there are few known causes of methylmalonic acidemia, all of which have only been reported in humans. Among these are dehydration, small intestinal bacterial overgrowth (SIBO), and inherited defects of the enzyme systems involved in metabolism of cobalamin and MMA.^{27,41,63,66} It cannot be excluded that some dogs included in this study were affected by one of these conditions. Dehydration may cause increased serum concentrations of both creatinine and MMA, while the effect on serum cobalamin concentration is unknown. Theoretically, SIBO may increase serum MMA concentrations if there is an overabundance of bacteria producing propionic acid, a precursor of D-methylmalonyl-CoA.⁶⁶ An excess of propionic acid could cause increased formation of MMA, and we cannot rule out that this may have occurred in some of the dogs. Additionally, SIBO may cause cobalamin deficiency because certain bacterial species utilize cobalamin present in the intestinal lumen and make it unavailable for absorption by the host.⁶⁷ Thus, SIBO may have a two-fold effect on serum MMA concentrations.

Little is known about inherited defects of enzymes involved in MMA metabolism in dogs. Those disorders that have been described are related to defects in cobalamin absorption and are generally accompanied by severe hypocobalaminemia.⁶⁸⁻⁷⁰ Thus, they would not explain increased serum MMA concentrations in the presence of normal serum cobalamin concentrations.

A defect in transcobalamin II could potentially cause increased serum MMA concentrations despite normal serum cobalamin concentrations, because transcobalamin II is the transport protein for cobalamin uptake by the cells.^{27,34} If delivery into the cells is hindered, cellular cobalamin deficiency may result, while serum cobalamin concentrations would remain within normal limits. In humans, measurement of holotranscobalamin, the biologically active fraction of cobalamin that is bound to transcobalamin, has been proposed instead of measuring serum cobalamin concentrations.⁷¹ Unfortunately, an assay for use in dogs is currently unavailable.

Conclusions - A large number of dogs with hypocobalaminemia as well as some dogs with normal serum cobalamin concentrations have metabolic evidence of cellular

cobalamin deficiency as determined by serum MMA concentration. It remains to be determined why some dogs have increased serum MMA concentrations despite serum cobalamin concentrations within the reference interval, and whether they should be supplemented with cobalamin. Supplementation with cobalamin may be justified because it has no known side effects and represents an affordable means of treatment. An investigation into the effect of cobalamin supplementation in dogs with increased serum MMA concentrations and both decreased and normal serum cobalamin concentrations is needed to better understand the relationship between serum cobalamin and MMA concentrations.

3. SERUM COBALAMIN AND METHYLMALONIC ACID CONCENTRATIONS IN DOGS WITH CHRONIC ENTEROPATHIES*

3.1 INTRODUCTION

The reported prevalence of hypocobalaminemia in dogs with chronic gastrointestinal disease ranges from 6% to 19%.^{1,32} Cobalamin deficiency may develop during chronic and severe small intestinal disease due to damage of mucosal receptors for the intrinsic factor (IF)-cobalamin complex, with subsequent reduced cobalamin absorption and, once cobalamin stores are depleted, resultant cobalamin deficiency.²⁶

Cobalamin deficiency in dogs is traditionally assessed by measuring the serum cobalamin concentration- a method that provides only a snapshot of the patient's current serum cobalamin status but cannot detect cellular cobalamin deficiency. A well-established method of evaluating the cellular cobalamin status is measurement of serum methylmalonic acid (MMA).^{72,73} Serum concentrations of MMA increase when tissue lacks adenosyl-cobalamin, a necessary co-factor for conversion of methylmalonyl-CoA to succinyl-CoA. Without cobalamin, the metabolism of methylmalonyl-CoA is shifted to an alternate pathway which yields MMA.³⁶ Therefore, increased serum MMA concentrations are considered a marker of cellular cobalamin deficiency.^{27,72,73}

Serum MMA concentrations may also be increased in patients with renal insufficiency and correlate with serum creatinine concentrations in humans.^{40,72} However, increases in serum MMA concentrations are generally only seen with serum creatinine concentrations >2.5 mg/dL, and even then, changes are considered minor in magnitude (up to 500 nmol/L).³⁹

A recent study comparing serum MMA and creatinine concentrations in more than 500 canine serum samples did not find a correlation between these two

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parameters.⁷³ Thus, it appears unlikely that minor variations in serum creatinine concentrations would significantly impact the serum MMA concentration. To rule out any potential impact of renal insufficiency on the serum MMA concentration, however, it may be advisable to evaluate serum creatinine concentrations whenever serum MMA concentrations are determined.

A recent study investigating cobalamin and MMA concentrations in more than 500 canine serum samples submitted to the Gastrointestinal Laboratory showed that 46% of dogs with hypocobalaminemia had evidence of cellular cobalamin deficiency, based on an increased serum MMA concentration.⁷³ Furthermore, a number of dogs with low normal serum cobalamin concentrations also had increased serum MMA concentrations, suggesting cobalamin deficiency on a cellular level despite a normal serum cobalamin concentration.⁷³ It was not possible to discriminate between different diseases among dogs to classify them into different groups (e.g. chronic gastrointestinal disease vs. other diseases, such as gastrointestinal neoplasia or exocrine pancreatic insufficiency).

Hypocobalaminemia has been found to be a risk factor for negative outcome in dogs with chronic gastrointestinal disease,¹ suggesting a clinical importance of cobalamin in affected dogs. However, the prevalence of cellular cobalamin deficiency in dogs with chronic gastrointestinal disease is currently unknown.

We hypothesize that hypocobalaminemia and cobalamin deficiency on a cellular level are common in dogs with chronic gastrointestinal disease. Thus, the aim of this study was to determine the proportion of dogs with chronic gastrointestinal disease that have hypocobalaminemia and/or methylmalonic acidemia.

3.2 MATERIALS AND METHODS

Patient enrollment and sample collection - In this prospective study, serum samples were collected from 69 dogs with chronic gastrointestinal disease. Dogs were eligible for enrollment if clinical signs of gastrointestinal disease (e.g., vomiting,

diarrhea, weight loss) were present for at least three weeks (at least one clinical sign had to be present to be considered for enrollment).

At the time the sample was obtained from the dog, written owner consent was obtained, and the primary veterinarian of the patient assessed the clinical activity index (canine chronic enteropathy clinical activity index, CCECAI).¹ This scoring index evaluates the dog's attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss, serum or plasma albumin concentration, presence of ascites or peripheral edema, and pruritus. Each parameter is given a score from "0" (normal) to "3" (severe change).

Dogs that had recently been treated with cobalamin were excluded from analysis. Furthermore, the canine serum trypsin-like immunoreactivity (cTLI) concentration was measured in all patient samples to test dogs for exocrine pancreatic insufficiency (EPI), another cause for cobalamin deficiency. Dogs with a subnormal serum cTLI concentration ($<5.7 \mu\text{g/L}$) were excluded. If gastrointestinal histopathology reports were available, results were reviewed to exclude dogs with gastrointestinal neoplasia.

Serum samples were also collected from 43 healthy, client-owned dogs to use as control dogs. At the time of blood collection, a brief physical examination was performed by a veterinarian, and all dogs appeared healthy. Additionally, owners of the dogs were asked to fill out a questionnaire to help exclude the presence of underlying diseases. The study was approved by the Clinical Research Review Committee of the College of Veterinary Medicine, Texas A&M University (CRR#09-06 and 08-30).

Serum creatinine measurement - Routine serum creatinine analyses were performed in all samples using an automated chemistry analyzer system^b with an enzymatic assay method.^c The reference interval for serum creatinine was 0.5–1.4 mg/dL. The intra-assay CV% was 0.79%, and the inter-assay CV% was 1.11%.

Serum cobalamin and methylmalonic acid measurement - Serum cobalamin concentrations were measured using a competitive binding chemiluminescence assay.^a Our laboratory's reference interval for this assay was 251-908 ng/L. The lower and

upper detection limits for the cobalamin assay were 150 and 1,000 ng/L, respectively. The intra-assay CV% was 8.9%, and the inter-assay CV% was 9.6%.

Serum MMA concentrations were measured using a stable isotope dilution gas chromatography/mass spectrometry (GC/MS) method, as described previously.^{33,73} Briefly, MMA was extracted from serum samples using an on-column^h liquid/liquid extraction method, followed by silylation with MTBSTFAⁱ for subsequent GC/MS analysis.³³ GC/MS separation and analysis was performed with a DB-1ms column^j using an Agilent 6890N gas chromatograph and 5975C mass selective detector^k as described elsewhere.³³ Assay performance was verified using standards prepared with pure MMA^d in serial dilution from 16,000 to 63 nmol/L. The reference interval for the MMA assay was 415-1,193 nmol/L, and the lower and upper detection limits were 63 and 16,000 nmol/L, respectively. Serum samples with MMA concentrations higher than 16,000 nmol/L were rerun at a dilution to allow measurement within the assay's linear range. Diluted sample concentrations were then back-calculated to obtain the actual sample concentration. MMA was quantified using the area under the curve for MMA and the internal standard (deuterated MMA).^e

Statistical analysis - Data were analyzed using GraphPad Prism 5.00.^f All data sets were analyzed for normal distribution of data using a D'Agostino & Pearson omnibus normality test. The mean (\pm SD), or the median and ranges of the data sets were calculated, as appropriate. Serum cobalamin and MMA concentrations were compared to a group of 43 healthy control dogs using a Mann-Whitney U test. A correlation between serum cobalamin and MMA concentrations, as well as between both serum parameters and the CCECAI in dogs with chronic gastrointestinal disease was evaluated using Spearman's rank correlation. A Spearman's rank correlation was also calculated for serum MMA and serum creatinine concentrations. A possible association between serum MMA concentrations and age, as well as duration of clinical signs was evaluated by Spearman's rank correlation as well as a Mann-Whitney U test. Statistical significance for all tests was set at $p < 0.05$.

3.3 RESULTS

Signalment of enrolled dogs - A total of 56 of the 69 dogs with gastrointestinal disease evaluated were eligible for enrollment. Of the 56 dogs, 29 were female (3 intact, 26 spayed) and 27 were male (4 intact, 23 castrated). The dogs' ages ranged from 0.4 to 13.0 years, with a mean (\pm SD) of 6.0 ± 3.3 years. There was no statistical difference in age between female and male dogs ($p=0.9743$).

Forty-six dogs were pure bred, with the following frequencies (n): Labrador Retriever (4), German Shepherd (3), Boxer (2), Cocker Spaniel (2), English Bulldog (2), Golden Retriever (2), Pitbull (2), Pointer (2), Standard Poodle (2), Yorkshire Terrier (2), Australian Shepherd (1), Basenji (1), Belgian Malinois (1), Bichon Frise (1), Border Collie (1), Boston Terrier (1), Cairn Terrier (1), Chesapeake Bay Retriever (1), Chinese Shar Pei (1), English Mastiff (1), French Bulldog (1), Irish Wolfhound (1), Jack Russell Terrier (1), Miniature Poodle (1), Miniature Schnauzer (1), Newfoundland (1), Pomeranian (1), Rhodesian Ridgeback (1), Soft-Coated Wheaten Terrier (1), Shiba Inu (1), Shih Tzu (1), Springer Spaniel (1), and Toy Poodle (1). The remaining 10 dogs were of mixed breed, with a mean (\pm SD) weight of 14.4 ± 10.7 kg (range: 2.6-31.5 kg).

The 43 healthy control dogs consisted of 23 spayed females and 20 males (4 intact, 16 castrated). The control dogs' ages ranged from 0.7 to 11.0 years, with a median of 3.0 years. There was no statistical difference in age between female and male dogs ($p= 0.4112$).

Of the control dogs, 29 were purebred, with the following breed frequencies: German Shepherd (3), Australian Shepherd (2), Beagle (2), Border Collie (2), Doberman Pinscher (2), American Pitbull Terrier (1), American Bulldog (1), Bloodhound (1), Blue Heeler (1), Boston Terrier (1), Chesapeake Bay Retriever (1), Welsh Corgi (1), Dachshund (1), English Springer Spaniel (1), Foxhound (1), Great Dane (1), Jack Russell Terrier (1), Labrador Retriever (1), English Mastiff (1), Neapolitan Mastiff (1), Rat Terrier (1), Rottweiler (1), Staffordshire Bull Terrier (1). Fourteen dogs were of mixed breed, with a mean (\pm SD) weight of 24.6 ± 7.2 kg (range: 12.0-34.2 kg).

Dogs with gastrointestinal disease were significantly older (median [range]: 6.1 [0.4-13] years) than healthy control dogs (median [range]: 3.0 [0.7-11.0] years; $p=0.0008$).

Clinical disease activity index - The median CCECAI for the 56 dogs was 6, with a range from 1.0-15.0.

Serum creatinine concentrations - The mean (\pm SD) serum creatinine concentration for the 56 dogs with gastrointestinal disease was 0.9 ± 0.3 mg/dL. Two of the 56 dogs had serum creatinine concentrations above the upper limit of the reference interval (1.7 and 1.8 mg/dL, respectively). One of these two dogs had an increased serum MMA concentration (3,543 nmol/L), and an undetectable serum cobalamin concentration (<150 ng/L). There was no correlation between serum MMA and serum creatinine concentrations ($p=0.5545$).

Serum cobalamin and MMA concentrations - Serum cobalamin concentrations in the 56 dogs with chronic gastrointestinal disease (median: 344 ng/L; range: 150-1,000 ng/L) were significantly lower than in a group of 43 healthy control dogs (median: 515 ng/L, range: 333-835 ng/L; $p=0.0011$; **Figure 3.1**). Twenty of the 56 dogs with gastrointestinal disease (36%) had cobalamin concentrations below the lower limit of the reference interval (<251 ng/L), and seven dogs (13%) had undetectable serum cobalamin concentrations (<150 ng/L).

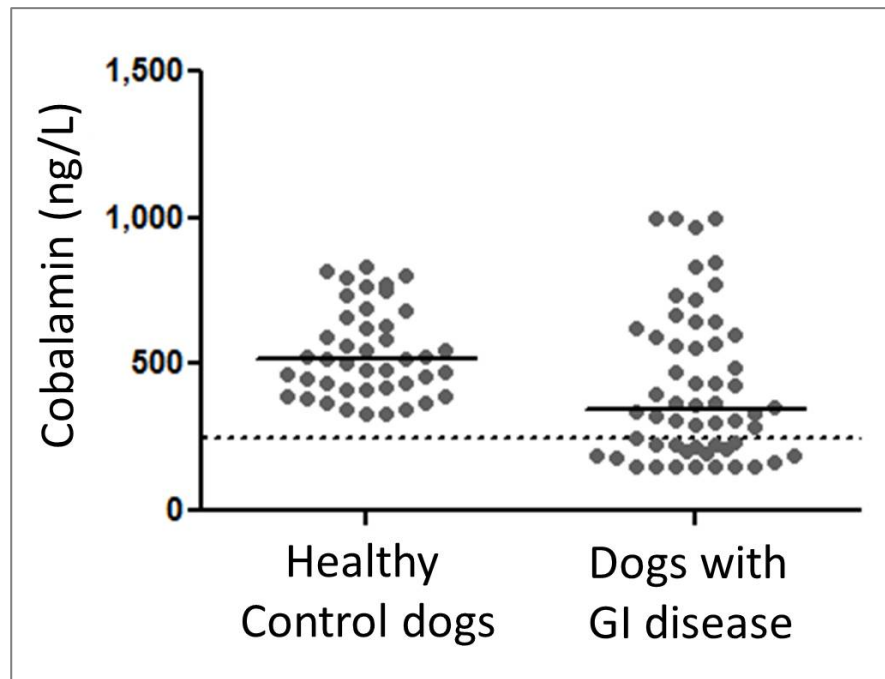


Figure 3.1 – Serum cobalamin concentrations in 43 healthy control dogs and 56 dogs with chronic enteropathies. The lower limit of the reference interval for the serum cobalamin concentration (251 ng/L) is depicted by the dotted line.

Median serum MMA concentration in dogs with chronic gastrointestinal disease was 741 nmol/L (range: 447-205,399 nmol/L), and serum MMA concentrations in dogs with chronic gastrointestinal disease were higher than in the group of 43 healthy dogs (median: 649 nmol/L, range: 228-1,253 nmol/L; $p=0.0360$; **Figure 3.2**). Five of 56 dogs with gastrointestinal disease (9%) had MMA concentrations above the upper limit of the reference interval ($>1,193$ nmol/L). All five of these dogs had undetectable serum cobalamin concentrations. Therefore, 25% of hypocobalaminemic dogs had increased serum MMA concentrations.

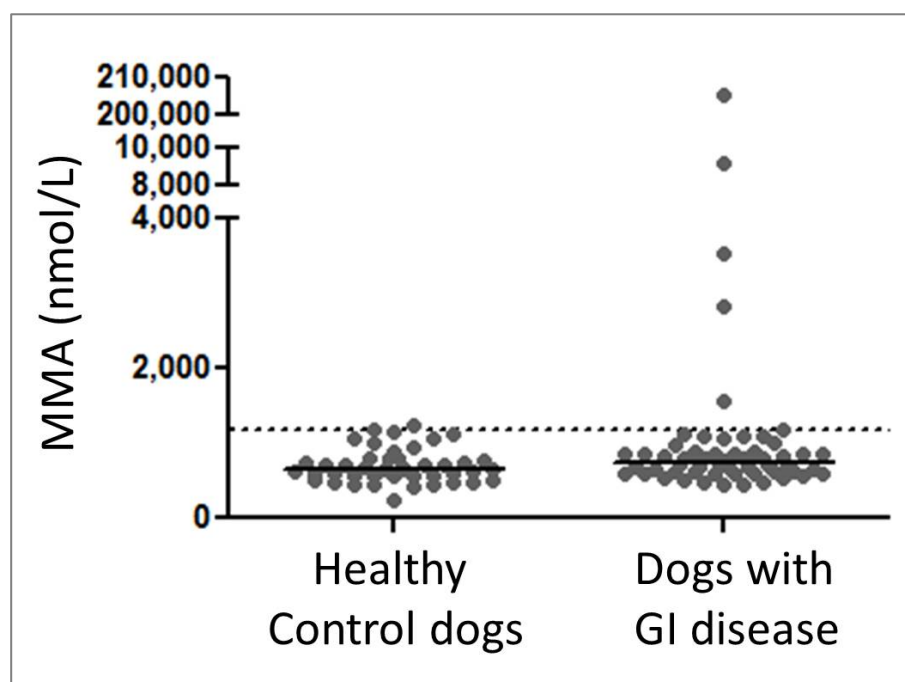


Figure 3.2 – Serum MMA concentrations in 43 healthy control dogs and 56 dogs with chronic enteropathies. The upper limit of the reference interval for the serum MMA concentration (1,193 nmol/L) is depicted by the dotted line.

When the two dogs with increased serum creatinine concentrations were removed from the analysis, serum MMA concentrations between dogs with gastrointestinal disease (median: 699 nmol/L, range: 447-205,399 nmol/L) and control dogs (median: 649 nmol/L, range: 228-1,253 nmol/L) no longer reached statistical significance ($p=0.0562$).

There was a moderate, significant negative correlation between serum cobalamin and MMA concentrations in dogs with chronic gastrointestinal disease (Spearman $r = -0.4495$, $p=0.0005$). No significant correlations were found between the CCECAI and serum cobalamin concentrations or the CCECAI and serum MMA concentrations.

There was no significant correlation between duration of clinical signs (median [range]: 6 [0.75-96] months) and serum MMA concentrations. No significant differences were found in duration of clinical signs between dogs that had increased serum MMA

concentrations (median [range]: 18 [4-84] months) and dogs with normal MMA concentrations (median [range]: 6 [0.75-96] months; $p=0.1763$).

Similarly, there was no association between age (median [range]: 6.1 [0.4-13] years) and serum MMA concentrations. Age was not significantly different between dogs with increased serum MMA concentrations (median [range]: 7.5 [1.8-10] years) and dogs with normal serum MMA concentrations (median [range]: 6.0 [0.4-13] years; $p=0.6874$).

3.4 DISCUSSION

The purpose of our study was to investigate the prevalence of hypocobalaminemia and cobalamin deficiency on a cellular level (as evidenced by increased serum MMA concentrations) in dogs with chronic gastrointestinal disease. To our knowledge, this is the first prospective study to determine serum MMA concentrations in dogs with chronic gastrointestinal disease.

The data show a prevalence of 36% for hypocobalaminemia in this group of dogs with chronic gastrointestinal disease, which is higher than previously reported for dogs with chronic gastrointestinal disease.^{1,25} Of 20 hypocobalaminemic dogs, 5 dogs (25%) also had an increased serum MMA concentration, suggesting cellular cobalamin deficiency. This number is lower than reported for dogs with hypocobalaminemia in another study,⁷³ and from the data shown in this study it is apparent that the differences in serum MMA concentrations between dogs with chronic gastrointestinal disease and healthy control dogs is only minor. Significance could no longer be detected once two dogs with a slightly increased serum creatinine concentration were removed from analysis. We chose to report data with these two dogs included, because data from our earlier study, as well as this study failed to indicate a correlation between serum creatinine and MMA concentrations in dogs. This is in contrast to humans where such a correlation has been reported.⁴⁰ In the previous study, dogs with a serum creatinine concentration as high as 3-4 mg/dL had normal serum MMA concentrations leading us

to believe that dogs would probably have to be in severe renal failure before it has a potential impact on serum MMA concentrations. Thus, the serum creatinine concentrations in the dogs reported here (1.7 and 1.8 mg/dL) probably did not contribute to their serum MMA concentrations.

In the aforementioned earlier study, 46% of dogs with a serum cobalamin concentration of less than 251 ng/L had increased serum MMA concentrations.⁷³ The prevalence observed in this investigation was only about half as high at 25%. This discrepancy is probably due to differences in populations that were sampled for these two studies. The earlier study used surplus serum samples that had been submitted to our laboratory, and no clinical data were available for dogs included in that study.

In the current study, dogs with conditions other than chronic gastrointestinal disease, such as EPI, or intestinal neoplasia, were excluded from analysis. Exocrine pancreatic insufficiency is one of the main causes of cobalamin deficiency in dogs; approximately 80% of EPI dogs develop a deficiency.²⁸ Intestinal neoplasia is also more likely to cause cobalamin malabsorption, and intestinal lymphoma has been associated with cobalamin deficiency in cats.^{31,74} For example, in this study, all four dogs that had been excluded from analysis due to intestinal neoplastic disease had hypocobalaminemia and/or methylmalonic acidemia. Therefore, it is conceivable that the described differences in the population sample between the two studies are partially responsible for the variation in results.

It is unclear why so many of the dogs with chronic gastrointestinal disease in this study had normal serum MMA concentrations despite the presence of low serum cobalamin concentrations. It is possible that the disease process in many of these dogs was not of sufficient chronicity to deplete cellular stores of cobalamin, which would be considered to be a prerequisite to the production of MMA.

An attempt was made to analyze the association between duration of clinical signs and serum MMA concentrations in this study. No significant correlation between these two parameters was found. While a trend towards longer duration of clinical signs in dogs with high serum MMA concentrations appeared to be present (median of 18

months vs. 6 months in dogs with normal serum MMA), it is difficult to evaluate these data statistically due to the low number of dogs in the group with high serum MMA concentrations (n=5), and no final conclusions should be drawn.

Half-life of cobalamin in healthy dogs is approximately two months (48-70 days), which is substantially shorter than in humans, in whom cobalamin half-life is approximately one year.^{31,75} These time frames are only applicable to healthy individuals, however. One study found a largely shortened half-life for cobalamin in cats with inflammatory bowel disease (5 days) when compared to healthy cats (12.8 days).³¹ It is likely that half-life of cobalamin is similarly decreased in dogs with gastrointestinal disease, due to reduced absorption while enterohepatic circulation with physiological excretion of cobalamin in bile is ongoing.

Data evaluating the temporal relationship between onset of hypocobalaminemia and development of methylmalonic acidemia in dogs are scarce. Studies in dogs with selective cobalamin malabsorption due to an inherited defect in the expression of proteins in the IF-cobalamin receptor complex (cubam [cubilin and amnionless]) have shown that serum MMA concentrations may be increased as early as two weeks after onset of hypocobalaminemia.⁷⁶ However, it is uncertain whether these findings also hold true for dogs with cobalamin malabsorption due to chronic gastrointestinal disease. Dogs with the inherited defect cannot absorb any cobalamin, whereas this is likely not the case in most dogs with gastrointestinal disease in which onset of cobalamin malabsorption may be gradual. Thus, further studies investigating the temporal relationship in dogs with chronic gastrointestinal disease may be warranted.

Median clinical activity index (CCECAI) was of moderate severity (median: 6) in dogs with gastrointestinal disease, with scores ranging from 1 to 15. There was no correlation between CCECAI and serum cobalamin or MMA concentrations, indicating that hypocobalaminemia and methylmalonic acidemia are not always associated with clinical disease severity. This can be explained by the fact that cobalamin deficiency in dogs with chronic gastrointestinal disease is caused by malabsorption of cobalamin due to damage to the mucosal receptors for the IF-cobalamin complex in the distal small

intestine.²⁶ Therefore, a patient with severe distal small intestinal disease is more likely to develop cobalamin deficiency than a patient with disease affecting other sites of the intestinal tract. We could not always determine which part of the intestine was primarily affected; but, after exclusion of dogs with EPI, it can be assumed that dogs with low serum cobalamin concentrations had at least some degree of ileal disease.³¹

Consequently, dogs with a high CCECAI yet normal serum cobalamin or MMA concentrations may have had more severe disease in other areas of the gastrointestinal tract. A potential limitation of our study is that some of the dogs may have received cobalamin prior to enrollment into our study, even though we asked treating veterinarians about supplementation and dogs were excluded from analysis if cobalamin supplementation was indicated as a treatment. In many cases, dogs were enrolled into the study through a referral clinic, but the patients had previously been seen by their primary care veterinarians. In those dogs, cobalamin may have been supplemented without being reported to us. This could have led to some discordance between the serum cobalamin concentrations and the clinical scores.

From the data presented here, we conclude that serum cobalamin deficiency is commonly seen in dogs with chronic gastrointestinal disease, but it does not always appear to be associated with a cellular cobalamin deficiency. The high prevalence of serum cobalamin deficiency in these dogs and the fact that hypocobalaminemia has previously been established as a risk factor for negative outcome in dogs with chronic gastrointestinal disease¹ leads us to believe that serum cobalamin concentrations should be measured in all dogs presenting with clinical signs of chronic gastrointestinal disease. Supplementation with cobalamin is strongly advised in all patients with a subnormal serum cobalamin concentration. It may also be advisable to supplement patients with low-normal serum cobalamin concentrations (<350 ng/L) if clinical signs of gastrointestinal disease are present. This recommendation is based on evidence that shows that cobalamin deficiency itself can cause gastrointestinal abnormalities, such as mucosal inflammation and villous atrophy, leading to nutrient malabsorption, including cobalamin.²⁶

Further studies are warranted to investigate the temporal relationship between the onset of serum cobalamin deficiency and cellular cobalamin deficiency in dogs with chronic gastrointestinal disease.

4. FECAL AND URINARY N-METHYLHISTAMINE CONCENTRATIONS IN DOGS WITH CHRONIC ENTEROPATHIES

4.1 INTRODUCTION

The role of mast cells in canine chronic gastrointestinal diseases is not well understood. However, mast cells are increasingly recognized as an important cell type for the innate and adaptive immune response.¹⁰ They express toll-like receptors, and have the ability to present antigens,¹⁰ which makes them a potentially significant part of the intestinal immune system. Furthermore, mast cells are capable of releasing a variety of inflammatory mediators, including histamine, tryptase, TNF- α , TGF- β , and both pro- and anti-inflammatory cytokines.⁴²

Histamine is primarily stored in mast cells, and only to a much lesser extent in other cell types.⁴⁸ It has been proposed that histamine may be used as a marker of mast cell degranulation.⁴² However, upon release from mast cells during degranulation, histamine is rapidly metabolized, making the measurement of histamine unreliable. N-methylhistamine (NMH) is a stable metabolite of histamine, which is generated from histamine by methylation via the histamine N-methyltransferase (HNMT) enzyme system.⁴⁹ N-methylhistamine can readily be measured in both fecal and urine samples, and an assay for use in dogs has recently been described and analytically validated.⁵⁶

NMH has been suggested as a marker for mast cell degranulation and gastrointestinal inflammation in humans with inflammatory bowel disease (IBD) and other chronic gastrointestinal diseases.^{43,50} Increased urinary NMH concentrations have been documented in human patients with active Crohn's disease and ulcerative colitis.^{43,50,51} Urinary concentrations of NMH were shown to correlate with endoscopic severity indices,⁴³ clinical disease activity,^{43,50} and C-reactive protein (CRP) concentrations.⁵¹ The correlation between urinary NMH concentrations and clinical disease activity suggests that mast cells become activated only during flare-ups, but do not release significant quantities of histamine at other times.⁵⁰ Thus, the NMH assay may provide a useful tool for monitoring disease activity.

Studies in dogs have shown increased fecal NMH concentrations in Norwegian Lundehunds and Soft Coated Wheaten Terriers with gastrointestinal disease.^{57,59} Interestingly, increased mast cell degranulation has also been implicated as a potential contributing factor to the development of protein-losing enteropathy and nephropathy in Soft Coated Wheaten Terriers.⁶⁰

Minimally invasive or noninvasive markers for gastrointestinal inflammation in dogs are currently lacking. For example, serum CRP concentrations have been found to be of only moderate clinical utility, and contradicting results have been observed in several studies.^{1,19,24} Measurement of analytes in fecal or urine samples would provide a means of non- or minimally invasive evaluation of gastrointestinal inflammation. If proven to be clinically useful, such markers could enhance the diagnosis and monitoring of clinical patients.

We hypothesize that fecal and urinary NMH concentrations reflect the degree of intestinal mucosal mast cell degranulation in dogs with chronic enteropathies, and could therefore serve as a marker of intestinal inflammation in these patients. Therefore, the aim of this study was to measure NMH concentrations in fecal and urine samples collected from dogs with chronic enteropathies and to compare them to those measured in healthy control dogs.

4.2 MATERIALS AND METHODS

Patient eligibility and enrollment - Dogs were eligible for enrollment into this prospective study if they had clinical signs of gastrointestinal disease (e.g., vomiting, diarrhea, weight loss) for at least three weeks (at least one clinical sign had to be present at the time to be considered for enrollment), and gastrointestinal parasitic disease was ruled out by fecal examination and/or treatment with anthelmintics. Dogs with known concurrent diseases were excluded. Dogs might have been pretreated, as long as all treatments were reported to the investigators upon enrollment to allow for subsequent classification and, if necessary, exclusion of the patient. Seventy dogs with chronic

gastrointestinal disease met initial inclusion criteria. If gastrointestinal histopathology reports were available, results were reviewed to exclude dogs with gastrointestinal neoplasia.

Written owner consent was obtained at the time the samples were collected. The study was approved by the Clinical Research Review Committee of the College of Veterinary Medicine, Texas A&M University (CRRC#09-06).

Sample collection - At enrollment, the dog's primary veterinarian assessed the clinical activity index for the dog (canine chronic enteropathy clinical activity index, CCECAI).¹ This scoring index evaluates the dog's attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss, serum or plasma albumin concentration, presence of ascites or peripheral edema, and pruritus. Each parameter was given a score from "0" (normal) to "3" (severe change). To assess clinical severity, the sum of all scores was calculated, and interpreted as follows: 0-3=insignificant disease; 4-5=mild disease; 6-8=moderate disease; 9-11=severe disease; and ≥ 12 =very severe disease.¹

One serum sample and three consecutive, naturally passed fecal samples were collected from each dog. If possible, a urine sample was also obtained by cystocentesis (preferred) or by free catch. Fecal samples were frozen immediately after collection. Serum and urine samples were stored at 4°C until shipment if they were shipped the same day, or they were frozen if they were shipped later. All samples were shipped to the laboratory overnight on ice. Routine serum analyses were performed immediately after arrival, and samples were stored at 4°C until analysis was complete. Fecal samples were stored at -20°C and extracted within one week. Fecal extracts and urine samples were stored at -80°C until NMH analysis.

Three consecutive fecal samples were also collected from 49 healthy, client-owned dogs to use as control dogs.

Serum routine chemistry analysis - Routine serum chemistry analyses were performed using an automated chemistry analyzer system.^b Patients with liver enzyme activities (ALP, ALT, AST, GGT) of a single enzyme higher than two-fold the

laboratory's reference interval, or with increased activities of more than one liver enzyme, were excluded from the study.

Assessment of gastrointestinal function and disease - Serum cobalamin and folate concentrations were measured using competitive binding chemiluminescence assays.^{al} Our laboratory's reference interval for the cobalamin assay was 251-908 ng/L and the lower and upper detection limits were 150 and 1,000 ng/L, respectively. The reference interval for serum folate concentration was 7.7-24.4 µg/L, and its lower and upper detection limits were 1.0 and 24.0 µg/L, respectively.

The serum canine trypsin-like immunoreactivity (cTLI) concentration was measured using a commercially available radioimmunoassay^m to test for exocrine pancreatic insufficiency (EPI). The reference interval for this assay was 5.7 to 45.2 µg/L. Dogs with a subnormal serum cTLI concentration (less than 5.7 µg/L) were excluded from the study.

Serum canine pancreatic lipase immunoreactivity (PLI) was measured as Spec cPL®.ⁿ The reference interval for this assay was 0-200 µg/L. Spec cPL concentrations higher than 400 µg/L were considered consistent with pancreatitis, and patients with Spec cPL concentrations >400 µg/L were excluded from the study. Concentrations of 201-399 µg/L were considered questionable.

Concentrations of C-reactive protein (CRP) in serum samples were measured using a commercially available ELISA assay kit.^o Our laboratory's reference interval for the CRP assay was ≤7.6 mg/L.

Fecal canine alpha₁-proteinase inhibitor (α₁-PI) concentrations were measured in all three consecutive fecal samples from each dog using a previously described radioimmunoassay (RIA).⁷⁷ Prior to analysis, a liquid extract of each fecal sample was prepared as previously described.⁷⁸ Briefly, fecal samples were diluted 1:5 in PBS-NBCS,^p followed by vigorous agitation on an automated vortex shaker for 20 min. Samples were subsequently centrifuged for 20 min at 2,100 x g and then filtered using a serum filter.^q The resultant supernatant was again centrifuged for 30 min at 10,000 x g. This second supernatant (= final fecal extract) was decanted and aliquots were stored at -

80°C until analysis. For the fecal α_1 -PI RIA, a further 1:200 dilution of the final fecal extract in RIA buffer^f was prepared.

To account for variability among the three fecal samples, both the 3-day mean fecal α_1 -PI concentration and the maximum concentration for the three days (3-day maximum) were calculated.⁷⁹ The reference intervals for the mean and maximum α_1 -PI were 2.2-13.9 $\mu\text{g/g}$ and 2.2-21.0 $\mu\text{g/g}$, respectively.⁷⁷

N-methylhistamine assay - N-methylhistamine was measured in fecal extracts and urine samples using stable isotope dilution gas chromatography/mass spectrometry (GC/MS), based on a previously reported method.^{49,56} For fecal NMH analysis, the same final fecal extract (1:5 dilution) as described for α_1 -PI analysis was used.

To allow for quantification of NMH, a known amount of trideuterated NMH (d3-NMH, 2.5 μL of a 200 $\mu\text{g/mL}$ solution) was added to 500 μL of liquid fecal extract or urine sample as an internal standard. After further addition of 500 μL of borate buffer,^s samples were vortexed thoroughly and applied to a silica solid-phase extraction column.^t The columns were washed with two 1 mL aliquots of ultrapure water^u and samples were eluted from the column with three 1 mL aliquots of acidified methanol.^v Eluates were dried under a stream of nitrogen, re-dissolved in 300 μL of 20% methanol in chloroform, and applied to a second silica column. The column was briefly washed with a 150 μL aliquot of 20% methanol in chloroform, followed by elution of the samples using four 1 mL volumes of methanol:chloroform:ammonium hydroxide (25:25:1, v/v). After eluates were dried under a stream of nitrogen, samples were derivatized for GC/MS analysis by acylation with pentafluoropropionic anhydride (PFPA).^w To each sample, 200 μL of ethyl acetate, 40 μL of pyridine, and 100 μL of PFPA were added. Samples were vortexed thoroughly, covered with thermoplastic laboratory film, and incubated at 64°C for 40 min. After derivatization, samples were evaporated once more under a stream of nitrogen. During the following partitioning step, 500 μL of Tris buffer and 1.5 mL of hexane were added to each sample and vigorously shaken on a vortex mixer for 1 min. After separation of the aqueous and hexane layers, the hexane layer was aspirated from the sample tube and placed into a clean sample tube. A second 1.5 mL hexane aliquot

was added to the Tris-sample tube, the partitioning step was repeated, and the combined hexane fractions were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 30 μ L of ethyl acetate, and transferred to a GC/MS autosampler vial.

The GC/MS analysis was performed using an Agilent 6890N GC and 5975C MSD^x with a dimethylpolysiloxane capillary column.^j Samples of 1 μ L volume were automatically injected into the GC inlet at 250°C in splitless mode, with an inlet pressure of 16.9 psi, and a total inlet flow of 19.6 mL/min. Helium was used as carrier gas at a constant column flow rate of 2.0 mL/min (velocity of 51 cm/sec). The initial column temperature was held at 50°C for 0.50 min, after which a temperature ramp was applied with a gradient of 20°C/min until 175°C was reached. The gradient was increased to 50°C/min up to a final temperature of 300°C, which was held for 2.00 min, resulting in a total run time of 11.25 min. The MS was operated in selected ion monitoring mode with ions at mass-to-charge ratios (m/z) of 417 and 420 for NMH and d3-NMH, respectively (dwell time 100 msec). The MS source temperature was 230°C, quadrupole temperature was 150°C, and transfer line temperature was 250°C. Assay performance was verified by use of standards with NMH concentrations ranging from 0 to 5,000 pg/ μ L.

Urinary NMH concentrations were normalized against urine creatinine concentrations and expressed in ng/mg creatinine. A urinary NMH concentration of >136 ng/mg creatinine was considered abnormal.⁵⁶ Fecal NMH concentrations were back-calculated for the wet weight of the fecal samples and expressed in ng/g feces. Similar to the fecal α_1 -PI assay, both 3-day mean and 3-day maximum concentrations were calculated for the fecal NMH assay. The reference intervals for the 3-day mean and maximum fecal NMH concentrations were ≤ 191 ng/g feces, and ≤ 334 ng/g feces, respectively.

Urine creatinine assay - Urine creatinine concentrations for normalization of urinary NMH concentrations were measured in all urine samples using an automated chemistry analyzer system^b with an enzymatic assay method.^c Urine samples were diluted 1:20 in purified water before analysis to ensure sample concentrations within the linear range of the assay (<30 mg/dL creatinine).

Statistical analysis - Data were analyzed using GraphPad Prism 5.00.^f All data sets were analyzed for normal distribution of data using a D'Agostino & Pearson omnibus normality test. Mean (\pm SD), or the median and ranges of the data sets were calculated, as appropriate. The CCECAI scores, though normally distributed, were reported as medians due to the categorical nature of the data. Fecal NMH concentrations were compared between dogs with gastrointestinal disease and healthy control dogs using a Mann-Whitney U test. Correlations between data sets were calculated using Spearman's rank correlation (r_s). Frequency distributions were analyzed using Fisher's exact test. To assess the relationship between fecal and urinary NMH concentrations and histological severity, inflammatory infiltrates were grouped as mild or moderate-severe, and outcomes were categorized as normal or increased NMH concentrations. This analysis was carried out separately for urinary and fecal NMH concentrations, and was also separated into analysis of gastric tissue only, duodenal tissue only, or both tissues combined. Statistical significance for all tests was set at $p < 0.05$.

4.3 RESULTS

Signalment of enrolled dogs - Forty-seven of the 70 dogs initially evaluated met the inclusion criteria and were included in the study. Of these, 24 were female (2 intact, 22 spayed), and 23 were male (3 intact, 20 castrated). The median age of the 47 dogs was 6.0 years, with a range from 0.4 to 12.0 years. There was no significant difference in age between females and males ($p = 0.755$).

Of the 47 dogs, 37 were purebred. The breeds most commonly seen were the German Shepherd (3), Labrador Retriever (3), Chinese Shar Pei (2), Cocker Spaniel (2), Golden Retriever (2), Pitbull (2), Pointer (2), and Yorkshire Terrier (2). All other purebred dogs were represented by only one member of their breed. Among these were a Boxer and a Soft Coated Wheaten Terrier. The remaining 10 dogs were of mixed breed, and included two Yorkshire Terrier crossbreeds.

The 49 control dogs comprised 22 females (1 intact, 21 spayed) and 27 males (4 intact, 23 castrated). The median age of the 49 dogs was 3.8 years (range: 0.5 to 17.3 years). There was no significant difference in age between control dogs and dogs with gastrointestinal disease ($p=0.106$). Of the 49 control dogs, 21 were mixed breed dogs and 28 were pure breeds. The most common breeds were Beagle ($n=3$), Dachshund (2), Pug (2), and Yorkshire Terrier (2). All other breeds were represented by a single dog each, among these one Boxer and a German Shepherd.

Clinical disease activity index and clinical signs - Median CCECAI for the 47 dogs was 6 (range: 1.0-14.0), which corresponds to moderate disease activity. The most frequently observed clinical signs were diarrhea with weight loss (8), followed by diarrhea only (7), vomiting with diarrhea (6), and vomiting with diarrhea, weight loss and inappetence (6). The remaining dogs had various combinations of the four above mentioned clinical signs.

Serum routine chemistry profile results - Notable abnormalities found on routine serum chemistry profiles were hypoproteinemia in 26 dogs (55%), and hypoalbuminemia in 14 dogs (30%). Hypocalcemia was noted in 14 dogs (30%), 11 of which were hypoalbuminemic and hypoproteinemic, and another three of which only were hypoproteinemic. There was a positive correlation between serum calcium and albumin concentrations (Spearman $r_s=0.8182$), and calcium and total protein concentrations ($r_s=0.7669$; $p<0.0001$ for both). Hypcholesterolemia was seen in 10 dogs (21%), and moderate positive correlations were also found between serum cholesterol concentrations and serum albumin ($r_s=0.4666$), total protein ($r_s=0.5095$), and calcium concentrations ($r_s=0.5108$; $p<0.001$ for all).

Assessment of gastrointestinal function and disease - For serum cobalamin concentration, only 45 of 47 dogs could be assessed, as two dogs had received cobalamin supplements prior to testing. Of these 45 dogs, 16 (34%) were hypocobalaminemic (<251 ng/L). Four of 47 dogs (9%) had a low serum folate concentration.

Five dogs (11%) had a serum Spec cPL® concentration within the equivocal range of the assay (201-399 µg/L), with the remainder of the dogs having Spec cPL concentrations within the reference interval.

Serum CRP concentrations were increased in 21/47 dogs (45%; median [range]: 15.6 mg/L [7.9-112.9]). Median (range) CRP concentration for all 47 dogs was 3.2 mg/L (0.1-112.9). There was a significant correlation between serum CRP concentrations and the CCECAI scores ($r_s=0.3382$, $p=0.020$).

Fifteen of the 47 dogs (32%) had increased 3-day mean fecal α_1 -PI concentrations, with a median of 7.2 µg/g feces for all 47 dogs (range: 2.2-402.0). Similarly, 16/47 dogs (34%) had increased 3-day maximum fecal α_1 -PI concentrations (median [range]: 9.2 µg/g feces [2.2-432.1]). All dogs with an increased 3-day mean also had increased 3-day maximum α_1 -PI concentrations. A moderate negative correlation was found between the fecal α_1 -PI concentration, and serum albumin concentration (3-day mean: $r_s=-0.3501$, $p=0.016$; 3-day max: $r_s=-0.3342$, $p=0.022$), as well as total protein concentration (3-day mean: $r_s=-0.4549$, $p=0.001$; 3-day max: $r_s=-0.4603$, $p=0.001$).

Fecal and urinary N-methylhistamine concentrations - Of 47 dogs, 9 (19%) had increased 3-day mean fecal NMH concentrations (median [range]: 2,465 ng/g [218-4,690]). Median 3-day mean fecal NMH concentration in all 47 dogs with gastrointestinal disease was 96 ng/g (range: 0-4,690), which was significantly higher than in control dogs (median [range]: 53 ng/g [9-252]; $p=0.026$; **Figure 4.1**).

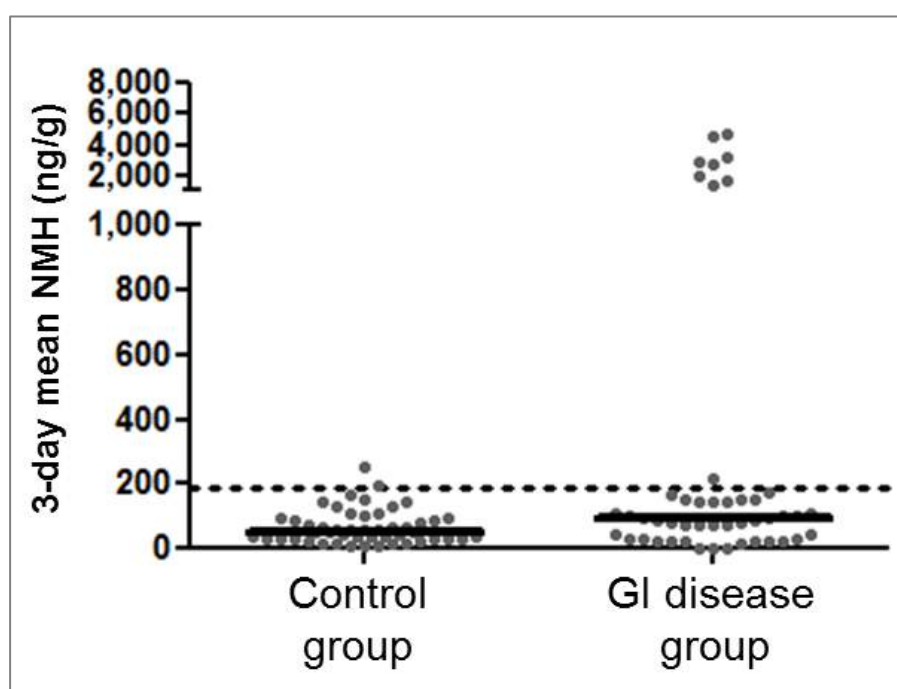


Figure 4.1 – 3-day mean fecal NMH concentrations in dogs with chronic enteropathies and healthy control dogs. 3-day mean fecal NMH concentrations were increased in 9 dogs (19%) with chronic enteropathies (median [range] in 9 dogs with increased NMH concentrations: 2,465 ng/g [218-4,690]). Overall medians in both groups are depicted by the solid black lines. The dotted line represents the upper limit of the reference interval (<191 ng/g feces).

The 3-day maximum fecal NMH concentrations were increased in 10/47 dogs (21%; median [range]: 2,777 ng/g [343-7,059]), which included the 9 dogs with an increased 3-day mean fecal NMH. Correspondingly, the 3-day maximum fecal NMH concentration in dogs with gastrointestinal disease (median [range]: 149 ng/g [0-7,059]) was significantly higher than in control dogs (median [range]: 83 ng/g [14-666]; $p=0.018$; **Figure 4.2**).

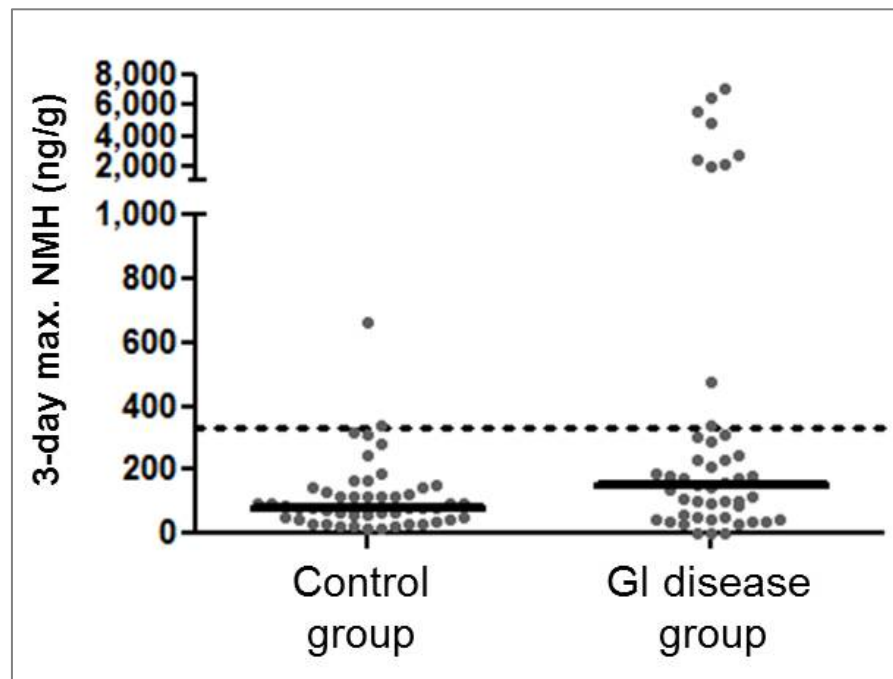


Figure 4.2 – 3-day maximum fecal NMH concentrations in dogs with chronic enteropathies and healthy control dogs. 3-day maximum fecal NMH concentrations were increased in 10 dogs (21%) with chronic enteropathies (median [range] in 10 dogs with increased NMH concentrations: 2,777 ng/g [343-7,059]). Overall medians in both groups are depicted by the solid black lines. The dotted line represents the upper limit of the reference interval (<334 ng/g feces).

Urine was available from 41/47 dogs (87%). Eleven of 41 dogs (27%) had increased urinary NMH concentrations. Three of these 11 dogs also had increased fecal NMH concentrations. Neither 3-day mean nor maximum fecal NMH concentration correlated with urinary NMH concentrations ($p=0.941$ and $p=0.609$, respectively).

A weak correlation was found between urinary NMH concentrations and serum CRP concentrations ($r_s=0.3121$, $p=0.047$; **Table 4.1**). However, no correlations were found between fecal NMH concentrations and the serum CRP concentrations, or any of the NMH concentrations and CCECAI scores. There was no correlation between fecal or urinary NMH concentrations and serum total protein or albumin concentrations, nor were dogs with increased NMH concentrations more likely to have low serum protein concentrations.

Data analysis was repeated using only those dogs in which gastrointestinal inflammation had been confirmed by histopathology, and from which complete sample sets were available (i.e., both fecal and urine samples available; $n=23$). Overall results were similar to those obtained with all 47 dogs included (see **Table 4.2** and **4.3**). However, the correlation between urinary NMH concentrations and serum CRP concentration, which was weak when all 47 dogs were included, was stronger when only dogs with confirmed gastrointestinal inflammation were included ($r_s=0.6546$, $p=0.001$; **Table 4.1**).

Analyses were also repeated after excluding dogs that had been pre-treated with either H₂-receptor antagonists, glucocorticoids, or both, in order to rule out a potential impact of these treatments on the outcome of the analysis. None of the dogs had received proton pump inhibitors. Analyses were done on both groupings of dogs, the 47 original dogs, and only those 23 dogs for which gastrointestinal inflammation had been confirmed histopathologically. Results were calculated after exclusion of dogs that received H₂-receptor antagonists (resulting in 37 and 18 untreated dogs for all dogs and the dogs with histopathology results, respectively), after exclusion of dogs that received glucocorticoids (42 and 21 dogs, respectively), as well as after exclusion of all pre-treated dogs (32 and 16 dogs, respectively; **Table 4.1**, **4.2** and **4.3**). Results showed no

large changes in outcome of the t-test comparison to control dogs. The overall percentage of dogs with increased fecal NMH concentrations increased after exclusions were made. However, the correlation between CRP and urinary NMH concentrations remained moderately strong in the histopathology group, even after removal of dogs that were treated with steroids or H₂ receptor antagonists, whereas the correlation between CRP and the CCECAI was no longer statistically significant once treated dogs were removed (**Table 4.1**).

Table 4.1 – Correlations of serum CRP concentrations with the CCECAI and urinary NMH concentrations. Correlations between serum CRP concentrations and the CCECAI, as well as urinary NMH concentrations in all 47 enrolled dogs, and all dogs not treated with histamine receptor antagonists and/or glucocorticoids. The Spearman correlation coefficient (r_s), as well as the p-value of the correlation are given for each analysis. The analysis was repeated to include only those dogs for which gastrointestinal inflammation had been confirmed histopathologically (n=23).

	Correlations				
	CRP vs. CCECAI			CRP vs. urinary NMH	
	n	Spearman r_s	p	Spearman r_s	p
<i>All dogs</i>	47	0.3382	0.020	0.3121	0.047
no HA treatment	37	-	ns	-	ns
no GC treatment	42	-	ns	0.3352	0.043
no HA or GC treatment	32	-	ns	-	ns
<i>Dogs with histopathology</i>	23	0.4270	0.042	0.6546	0.001
no HA treatment	18	-	ns	0.6164	0.006
no GC treatment	21	-	ns	0.6415	0.002
no HA or GC treatment	16	-	ns	0.5916	0.016

HA=histamine receptor antagonist; GC=glucocorticoids; ns=not significant

Table 4.2 – Comparison of 3-day mean fecal NMH concentrations. Comparison of 3-day mean fecal NMH concentrations in all 47 enrolled dogs, and of all dogs not treated with histamine antagonists and/or glucocorticoids. Percentages of dogs with increased fecal NMH concentrations (with medians and ranges) are given for each group. The analysis was repeated to include only those dogs for which gastrointestinal inflammation had been confirmed histopathologically (n=23).

	3-day mean fecal NMH concentration [ng/g feces]					
				Dogs with increased NMH		
	n	Median (range)	t-test p*	n	%	Median (range)
<i>All dogs</i>	47	96 (0-4,690)	0.026	9	19%	2,465 (218-4,690)
no HA treatment	37	97 (0-4,690)	0.006	8	22%	2,665 (218-4,690)
no GC treatment	42	98 (0-4,690)	0.005	8	19%	2,666 (218-4,690)
no HA or GC treatment	32	104 (14-4,690)	0.001	7	22%	2,598 (218-4,690)
<i>Dogs with histopathology</i>	23	109 (0-4,515)	0.015	6	26%	2,665 (1,728-4,515)
no HA treatment	18	126 (0-4,515)	0.008	6	33%	2,665 (1,728-4,515)
no GC treatment	21	109 (0-4,515)	0.010	5	24%	2,598 (1,728-4,515)
no HA or GC treatment	16	126 (14-4,515)	0.004	5	31%	2,598 (1,728-4,515)

HA=histamine receptor antagonist; GC=glucocorticoids

*p-value of the t-test comparison to healthy control dogs: control dog median (range) for 3-day mean was 53 ng/g (9-252).

Table 4.3 – Comparison of 3-day maximum fecal NMH concentrations. Comparison of the 3-day maximum fecal NMH concentrations in all 47 enrolled dogs, and all the dogs not treated with histamine antagonists and/or glucocorticoids. Percentages of dogs with increased fecal NMH concentrations (with medians and ranges) are given for each group. The analysis was repeated to include only those dogs for which gastrointestinal inflammation had been confirmed histopathologically (n=23).

	3-day maximum fecal NMH concentration [ng/g feces]					
				Dogs with increased NMH		
	n	Median (range)	t-test p*	n	%	Median (range)
<i>All dogs</i>	47	149 (0-7,059)	0.018	10	21%	2,777 (343-7,059)
no HA treatment	37	160 (0-7,059)	0.005	9	24%	2,907 (343-7,059)
no GC treatment	42	167 (0-7,059)	0.003	9	21%	2,733 (343-7,059)
no HA or GC treatment	32	178 (27-7,059)	<0.001	8	24%	3,037 (343-7,059)
<i>Dogs with histopathology</i>	23	177 (0-7,059)	0.017	7	30%	2,907 (343-7,059)
no HA treatment	18	172 (0-7,059)	0.017	7	39%	2,907 (343-7,059)
no GC treatment	21	177 (0-7,059)	0.017	6	29%	3,037 (343-7,059)
no HA or GC treatment	16	172 (27-7,059)	0.010	6	38%	3,037 (343-7,059)

HA=histamine receptor antagonist; GC=glucocorticoids

* p-value of the t-test comparison to healthy control dogs: control dog median (range) for 3-day maximum was 83 ng/g (14-666).

Frequency distribution analysis revealed a significant association between urinary NMH concentrations and severity of mucosal inflammation in the duodenum ($p=0.008$, **Figure 4.3**).

In contrast, no significant association of fecal NMH concentration and mucosal inflammatory severity was found either for the stomach or the duodenum.

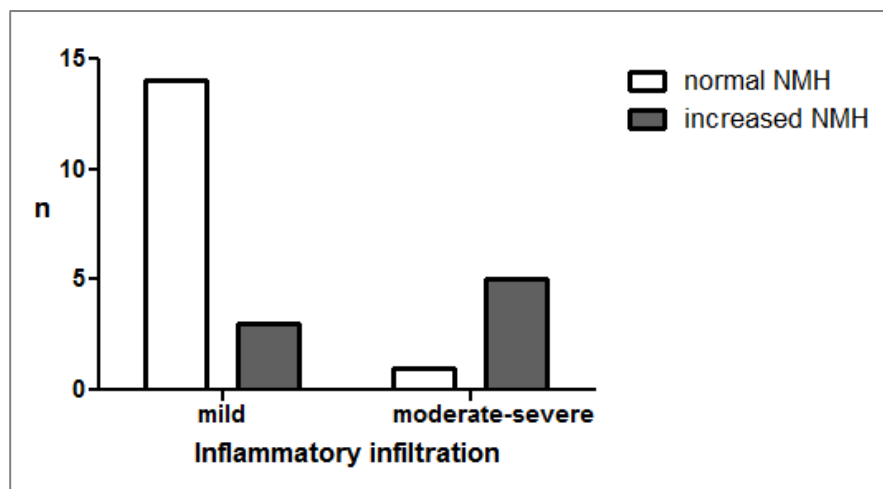


Figure 4.3 – Comparison of urinary NMH concentrations with mild versus moderate-severe gastrointestinal inflammation in the duodenum. This figure shows the comparison of urinary NMH concentrations with the degree of inflammation in the duodenal mucosa ($p=0.008$). Dogs with urinary NMH concentrations below the upper limit of the control range (≤ 136 ng/mg creatinine) are shown in white, dogs with increased urinary NMH concentrations are shown in dark grey.

4.4 DISCUSSION

To our knowledge, this is the first prospective study to investigate fecal and urinary NMH concentrations in a large group of dogs with chronic gastrointestinal disease. Preliminary data in Norwegian Lundehunds and Soft Coated Wheaten Terriers have been reported,^{57,59} but our goal was to investigate a larger group of dogs representative of a typical clinical patient set, instead of restricting our patient group to dogs of breeds known to have a higher prevalence of chronic enteropathies.

The aim of this study was to determine if fecal or urinary NMH concentrations are increased in dogs with chronic gastrointestinal disease, and whether measuring NMH in fecal or urinary specimens could serve as a useful marker of inflammation.

Results demonstrated that fecal and urinary NMH concentrations were increased in 21% (feces) to 27% (urine) of dogs with chronic gastrointestinal disease. This increase is indicative of mast cell degranulation, and suggests involvement of mast cells in the disease process. However, unlike in humans with IBD, in whom urinary NMH concentrations correlate with the clinical disease activity index and serum CRP concentration, no correlation could be observed for the 47 dogs in this study for either urinary or fecal NMH concentrations in regards to the CCECAI.

There was a weak correlation between urinary NMH concentration and CRP concentration when all 47 dogs were analyzed. In human patients, the serum CRP concentration represents an important measure for evaluation of disease activity in chronic gastrointestinal inflammation because of its short half-life.⁸⁰ Half-life of CRP in dogs is assumed to be equally short,⁸¹ and its use as a marker of disease activity and severity in dogs with chronic gastrointestinal disease has been evaluated in several studies, with varying results.^{1,19,24} In our study, the clinical activity index correlated with serum CRP concentrations, but not to the NMH concentrations. This suggests that NMH may not be a good indicator of disease activity in dogs with chronic gastrointestinal disease, as measured by the CCECAI.

Interestingly, when analysis was reduced to 23 dogs with histologically confirmed gastrointestinal inflammation, the correlation between urinary NMH and

serum CRP concentrations was significantly stronger than in the group including all 47 dogs. The serum CRP concentration in these 23 dogs was higher (median [range]: 8.0 mg/L [0.1-112.9]) than in the group of all 47 dogs (median [range]: 3.2 mg/L [0.1-112.9]). It is possible that the 23 dogs had more severe gastrointestinal inflammation, representing a different disease group than the remaining dogs, which may have led to a stronger association between the parameters investigated.

Comparison of the grade of histological inflammation (mild vs. moderate-severe) with urinary or fecal NMH concentrations revealed a significant association between urinary NMH concentrations and the grade of inflammation in the duodenal, but not the gastric mucosa. This suggests that urinary NMH concentrations may be more accurate for detection of intestinal than gastric inflammation. Fecal NMH concentrations did not have any association with mucosal inflammation. This observation is in agreement with studies in humans with IBD, in whom urinary NMH concentrations are a more accurate indicator of disease severity than fecal NMH concentrations.

One limitation of our study is a lack of follow-up data; therefore it was not always possible to designate dogs to specific subgroups, such as food-, antibiotic-, or steroid-responsive disease. It is conceivable that better characterization of subgroups could have identified an association between increases in NMH concentrations and a specific disease group. Follow-up data was available for a small number of dogs that did not allow for statistical analysis. Based on those dogs, no association between NMH concentrations and any treatment response could be detected. However, follow-up data on a larger number of dogs may provide a different outcome.

Another limitation is that some of the dogs had been pre-treated with glucocorticoids and/or antihistamines, and the effect this may have had on fecal or urinary NMH concentrations is unknown. We therefore modified our analysis to exclude dogs that had received glucocorticoids and/or antihistamine treatment (e.g. H₂ blockers), and repeated all statistical tests. No profound changes in statistical outcome were found, leading us to believe that these treatments did not have a significant impact on our results.

All dogs in this study were enrolled either with newly presenting disease, or with ongoing clinical signs despite prior treatment (i.e., treatment failures), suggesting that if they had received pre-treatment, the therapeutic attempts were not successful in treating the underlying disease process. Regardless, a potential influence of treatments on mast cell mediated inflammation cannot be excluded.

For future studies, treatment trials with different classes of antihistamines (H_1 blockers vs. H_2 blockers), as well as mast cell stabilizers (e.g. cromolyn sodium, ketotifen) in dogs with increased NMH concentrations may be warranted. Preliminary data in Soft Coated Wheaten Terriers with PLE have shown some therapeutic success in dogs resistant to traditional therapeutic intervention when treated with cromolyn sodium.⁸² Increased fecal NMH concentrations have been found in Soft Coated Wheaten Terriers with gastrointestinal disease, and this finding is intriguing.

To our knowledge, this was the first study to simultaneously evaluate fecal and urinary N-methylhistamine concentrations in any species. Studies in humans have evaluated either urinary^{43,50} or fecal NMH concentrations,⁸³ but no comparison has been made between the two sample types at the same time. In our study, fecal and urinary NMH concentrations did not correlate with each other, and only three of 11 dogs with increased urinary NMH concentrations had simultaneously increased fecal NMH concentrations. Due to lack of comparable studies in human medicine, it is unknown whether this result may be due to our specific group of dogs, or whether fecal and urinary NMH concentrations in general do not tend to correlate with each other.

Studies in humans, however, have shown that urinary NMH concentrations tend to be a better indicator of clinical disease activity in IBD patients than fecal NMH concentrations.⁴³ There may be several reasons for this observation.

First, histamine can be metabolized either via diamine oxidase to imidazole acetic acid, or, as described here, via histamine N-methyltransferase (HNMT) to NMH. Diamine oxidase is released from villous epithelial cells via intracellular vesicles, and then acts as an extracellular enzyme.⁸⁴ Within the gastrointestinal tract, it presumably converts primarily food-derived, intraluminal histamine.⁸⁵ Histamine N-

methyltransferase on the other hand, is a cytosolic enzyme with intracellular action,⁸⁵ and it has been shown that the vast majority of endogenously released histamine is metabolized via the HNMT pathway.^{43,86} It is possible that histamine released from mast cells that are located closer to the mucosal surface may be partially subjected to metabolism by diamine oxidase, because a portion of it may enter the intestinal lumen before it has been converted to NMH. This scenario could produce some discordance in fecal versus urinary NMH concentrations.

Second, distribution of mast cells within intestinal layers could lead to a similar disagreement. Histamine released by mast cells in the lamina propria or submucosa is converted to NMH, and may subsequently enter the blood or lymphatic system to be excreted in the urine, whereas NMH originating from mast cells closer to the mucosal surface may be more likely to be released into the intestinal lumen. If more mast cells are located towards the lamina propria and submucosa, versus the mucosa, an increase in NMH concentrations may more likely be seen in the urine, rather than the feces. An investigation of the distribution of mast cells in canine gastrointestinal biopsies in regards to fecal and urinary NMH concentrations is ongoing, and may help answer some of these questions.

Lastly, it is not known to what extent an uptake of NMH along the gastrointestinal tract with subsequent urinary excretion may occur, and how this may be affected by disease location within the gastrointestinal tract.

In summary, lack of correlation between NMH concentrations and the clinical activity index suggests that NMH may not be a good marker for clinical disease activity in dogs. However, these data show that some dogs with chronic gastrointestinal disease have increased fecal and/or urinary NMH concentrations, which indicates increased mast cell activity and involvement of mast cells in the disease process. Therefore, urinary and/or fecal NMH concentrations may possibly function as an independent marker of inflammation. Due to the detected association to serum CRP concentrations and severity of mucosal inflammation, urinary NMH concentrations may be a more suitable marker of intestinal inflammation than fecal NMH concentrations, which would be in agreement

with studies in human patients. Future studies are warranted to further characterize the role of mast cell mediated inflammation in dogs with chronic gastrointestinal disease. Also, evaluation of the potential benefit of alternative treatment approaches targeting mast cell stabilization and/or inhibition of mast cell mediator action may be facilitated if NMH can be used as a marker of mast cell degranulation.

5. SUMMARY AND CONCLUSION

5.1 SUMMARY

Diagnosis and management of chronic enteropathies in dogs often remains a challenge. Non-invasive laboratory tests are lacking, and a diagnosis is generally made based on exclusion of other possible disease processes.

Regardless of the type of enteropathy, assessment of clinical disease activity can be useful at initial diagnosis and each time the patient is re-evaluated, to ensure adequate therapy is initiated and appropriately modified. Clinical activity indices for use in dogs with chronic enteropathies have been proposed,^{1,24} but few laboratory tests have aided in the assessment of these patients. Serum or plasma albumin concentration is an objective parameter that is factored into the CCECAI, increasing the score if the albumin concentration is below the lower limit of the reference interval.¹ Also, while the serum cobalamin concentration is not taken into account for calculation of the clinical activity index, decreased serum concentrations of cobalamin have been associated with a negative outcome.¹ Correlation between serum CRP concentration and clinical activity has been reported in dogs with at least moderate disease,²⁴ but other studies have not reproduced this finding. Thus, identification of other laboratory parameters that may be useful as markers of active disease, or that could aid in assessment of patients with chronic enteropathies is desirable.

This study was designed to characterize two separate aspects of canine chronic enteropathies. One was to investigate the prevalence of cobalamin deficiency on a cellular level in dogs with chronic gastrointestinal disease by measurement of serum MMA concentration. The purpose of this investigation was to determine if dogs with evidence of cobalamin deficiency could be identified that would have gone undetected if only the serum cobalamin concentration had been measured. Based on the fact that hypocobalaminemia is a risk factor for negative outcome in dogs with chronic enteropathies, identification of dogs with cobalamin deficiency on a cellular level may be of clinical importance. The second objective of the study was to investigate the utility

of measuring fecal and urinary NMH concentrations as novel markers of intestinal inflammation and clinical activity in dogs with chronic enteropathies.

First, in order to determine the relationship between serum MMA and cobalamin concentrations in dogs, MMA concentrations were measured in a large sample set of 555 canine serum samples with serum cobalamin concentrations ranging from undetectable (<150) to 1,000 ng/L. To allow for interpretation of the serum MMA concentrations, a reference interval for canine serum MMA concentrations was established based on 43 healthy control dogs, and was determined as 415-1,193 nmol/L.

Results showed that 46% of hypocobalaminemic dogs (<251 ng/L) had increased serum MMA concentrations. Not unexpectedly, this proportion was even higher (63%) in dogs with undetectable serum cobalamin concentrations. Increased serum MMA concentrations (i.e., 16%-19% increase) were also seen in dogs with serum cobalamin concentrations as high as 350-450 ng/L, indicating that some dogs with normal serum cobalamin concentrations also have evidence of cellular cobalamin deficiency. This finding was interesting, as it suggested cellular cobalamin deficiency without concurrent hypocobalaminemia in a subset of the dogs evaluated. One factor known to increase serum MMA concentrations in the face of normal serum cobalamin concentrations is renal insufficiency. In humans, serum creatinine concentrations have been found to correlate with serum MMA concentrations. In this study, serum creatinine concentrations were measured in all serum samples for which sufficient serum was available (542/555), and concentrations were increased (>1.4 mg/dL) in 10% of samples. However, no influence of serum creatinine concentration on serum MMA concentration could be detected by regression analysis. Thus, it is unlikely that renal insufficiency contributed to the increased serum MMA concentrations observed in these dogs.

Further studies are needed to investigate whether dogs with increased serum MMA concentrations and normal serum cobalamin concentrations would benefit from cobalamin supplementation. For example, a trial could be carried out by supplementing these dogs with cobalamin. If cobalamin supplementation results in a significant decrease in serum MMA concentration, it is highly likely that the dog had cellular

cobalamin deficiency. If that were the case, a recommendation to treat dogs that have an increased serum MMA concentration in the face of normocobalaminemia with cobalamin supplementation may be warranted. Supplementation with cobalamin may be justified regardless, because it has no known side effects and represents an affordable means of treatment.

To investigate the prevalence of cobalamin deficiency on a cellular level in dogs with chronic enteropathies, serum samples were collected from 56 dogs, and cobalamin and MMA concentrations were measured in all samples. Hypocobalaminemia was found in 36% of these dogs, whereas methylmalonic acidemia was detected in only 9% of these dogs, all of which had undetectable serum cobalamin concentrations. Thus, 75% of hypocobalaminemic dogs had serum MMA concentrations within the reference interval, and only 25% had increased MMA concentrations.

This finding is in contrast to the almost 50% of hypocobalaminemic dogs with methylmalonic acidemia reported in the previous study.⁷³ A difference between the two study populations is likely responsible for this discrepancy. Our previous study utilized surplus serum samples, and no clinical data was available for the dogs. For the second study, only dogs with chronic enteropathies were enrolled, and those with gastrointestinal neoplasia and EPI were excluded, which likely produced a very different sample set in regards to etiologies, which may not be comparable to the previous study. However, it is still unclear why so many dogs with chronic gastrointestinal disease and hypocobalaminemia had normal serum MMA concentrations. One possible explanation is that the disease process in dogs with normal MMA concentrations may not have been of sufficient chronicity to cause cellular depletion of cobalamin and thus production of MMA. Therefore, the association between duration of clinical signs and serum MMA concentrations was explored. While there appeared to be a trend towards longer duration of clinical signs in dogs with increased serum MMA concentrations, no significant correlation was detected. Data on the temporal relationship between the onset of hypocobalaminemia and methylmalonic acidemia are scarce. Studies in dogs with selective cobalamin malabsorption due to a receptor defect indicate that serum

methylmalonic acid concentrations increase within as little as two weeks after onset of cobalamin deficiency. However, it is uncertain whether these findings are relevant for dogs with cobalamin malabsorption due to gastrointestinal disease, and studies investigating this temporal relationship in dogs with chronic gastrointestinal disease may be warranted.

Based on these data, it can be concluded that serum cobalamin deficiency is common in dogs with chronic gastrointestinal disease, but that it is not always associated with cellular cobalamin deficiency. The high prevalence of cobalamin deficiency in the dogs studied here suggests that serum cobalamin concentrations should be measured in all dogs with clinical signs of chronic gastrointestinal disease, to ensure that cobalamin supplementation can be initiated if necessary. This investigation did not identify any dogs with cellular cobalamin deficiency that did not also have concurrent hypcobalaminemia.

For the second part of the study, fecal and urinary NMH concentrations were measured in dogs with chronic enteropathies to investigate whether NMH could serve as a useful marker of inflammation and may aid in assessment of clinical activity in dogs with chronic enteropathies. Increased NMH concentrations in urine or fecal samples are indicative of mast cell degranulation, and therefore involvement of mast cells in the gastrointestinal disease process. Results showed increased fecal NMH concentrations in 21% of dogs, and increased urinary NMH concentrations in 27% of the dogs enrolled.

While urinary NMH concentrations in human patients with IBD correlate to the clinical activity index, no correlation was observed for the dogs in this study for either urinary or fecal NMH concentrations. A correlation between urinary NMH concentrations and serum CRP concentrations was found, which mirrors studies in human patients, in whom CRP and urinary NMH concentrations also correlate. This correlation became significantly stronger when the sample size was reduced to only include those dogs with histopathologically confirmed gastrointestinal inflammation. It is not unexpected to find this result, because availability of histopathological

confirmation of inflammation yields an improved case definition and a more closely defined group of patients.

Additionally, analysis of frequency distributions for the histological grade of inflammation (mild vs. moderate-severe) compared to urinary and fecal NMH concentrations showed a significant association between urinary NMH concentrations and the grade of inflammation in the duodenum. This suggests that urinary NMH concentrations may be more accurate for detection of intestinal, rather than gastric inflammation. Further studies are needed to determine the degree of mast cell involvement in gastric and duodenal inflammation in dogs. Fecal NMH concentrations were not associated with mucosal inflammation. This finding is in agreement with studies in human patients with IBD, in whom urinary NMH concentrations have been found to be a more accurate indicator of disease severity than fecal NMH concentrations.

Because this was the first investigation to simultaneously evaluate fecal and urinary N-methylhistamine concentrations in any species, it is unknown whether the lack of correlation between urinary and fecal NMH concentrations was due to our specific group of dogs, or whether they do not tend to correlate with each other in general. However, based on the studies in humans with IBD mentioned above, it is likely that urinary NMH concentrations are a better indicator of clinical disease activity and inflammation than fecal NMH concentrations, and this may be similar in dogs.

Modification of our analysis to exclude dogs that had been pre-treated with glucocorticoids and/or antihistamines prior to enrollment revealed no major changes in statistical outcomes for the parameters investigated. Treatments administered were likely given for varying lengths of time and an exact analysis of the effect of either H2 blockers or glucocorticoids would be difficult. It is possible that more NMH-positive dogs may have been identified if all dogs had been untreated, because a potential influence of treatments on mast cell mediated inflammation cannot be excluded; further studies are necessary to investigate the effect of antihistamine or glucocorticoid treatment on fecal and urinary NMH concentrations in dogs.

5.2 CONCLUSIONS

In conclusion, these data demonstrate that hypocobalaminemia is common in dogs with chronic gastrointestinal disease, but is not always associated with cobalamin deficiency on a cellular level. In this investigation of dogs with chronic enteropathies, no dogs were identified with cellular cobalamin deficiency that did not also have concurrent hypocobalaminemia.

Furthermore, some dogs with chronic gastrointestinal disease have increased fecal and/or urinary NMH concentrations, suggesting increased mast cell degranulation and involvement of mast cells in the disease process. No correlation of fecal or urinary NMH concentration to the clinical activity index was found, suggesting that NMH may not be a good marker for clinical disease activity as determined by CCECAI. However, urinary NMH concentrations may have clinical utility based on their correlation to serum CRP concentrations and an association with the severity of intestinal mucosal inflammation. Fecal NMH concentrations did not correlate with serum CRP concentrations or severity of inflammation. Future studies are warranted to further characterize the role of mast cell mediated inflammation in dogs with chronic gastrointestinal disease. Measurement of urinary and fecal NMH concentrations may be a useful adjunct marker in these studies.

ENDNOTES

- ^a Immulite 2000 Vitamin B12, Siemens Healthcare Diagnostics Inc., Deerfield, Ill.
- ^b Sirrus Clinical Chemistry Analyzer, Stanbio Laboratory, Boerne, Tex.
- ^c Direct Creatinine LiquiColor Test, Stanbio Laboratory, Boerne, Tex.
- ^d Methylmalonic acid, Sigma-Aldrich, St. Louis, Mo.
- ^e Methyl-d3-malonic acid, CDN Isotopes, Pointe-Claire, QC, Canada
- ^f GraphPad Software, San Diego, CA
- ^g JMP 8.0.2, SAS Institute, Inc. s
- ^h Chem Elut Cartridges, 1 mL, unbuffered, Agilent Technologies, Santa Clara, Calif.
- ⁱ MTBSTFA, #TS-48920, Thermo Fisher Scientific, Waltham, Mass.
- ^j DB-1ms #122-0132, 30 m length, 0.25 mm diameter, 0.25 µm film thickness, Agilent Technologies, Santa Clara, Calif.
- ^k Agilent Technologies, Santa Clara, Calif.
- ^l Immulite 2000 Folic Acid, Siemens Healthcare Diagnostics Inc., Deerfield, Ill.
- ^m Double Antibody Canine Trypsin-Like Immunoreactivity (TLI), Siemens Medical Solutions Diagnostics, Los Angeles, Calif.
- ⁿ IDEXX Laboratories, Westbrook, Maine
- ^o PHASE™ EIA Canine CRP Assay, Tridelata Development, Ltd., Maynooth, County Kildare, Ireland
- ^p PBS-NBCS: phosphate-buffered saline (BupH™, Thermo Fisher Scientific, Rockford, Ill.) with 5% (v/v) newborn calf serum (Sigma-Aldrich, St. Louis, Mo.), 1% (v/v) Triton X-100 (Surfact-Amps® X-100, Thermo Fisher Scientific, Rockford, Ill.), and 0.25 mM thimerosal (Sigma-Aldrich, St. Louis, Mo.)
- ^q Fisherbrand IB Model Serum Filters, Fisher Scientific Inc., Pittsburgh, Pa.
- ^r RIA buffer (pH 7.5): 0.05 M sodium phosphate, 0.02% (w/v) sodium azide, 0.5% (w/v) BSA
- ^s Sodium tetraborate decahydrate 10 mM, pH 9.0, Sigma-Aldrich, St. Louis, Mo.
- ^t SepPak®Vac Silica, 1cc, 100 mg, Waters Corporation, Milford, Mass.

^u Burdick & Jackson® water, Honeywell Burdick & Jackson, Muskegon, Mich.

^v 0.1 M HCl in methanol

^w Pentafluoropropionic anhydride, Sigma-Aldrich, St. Louis, Mo.

^x Agilent 6890N gas chromatograph and 5975C mass selective detector, Agilent Technologies, Santa Clara, Calif.

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